DEVELOPMENT OF A CARBON DIOXIDE GENERATION AND DELIVERY SYSTEM FOR CARBON-EFFICIENT CULTIVATION OF MICROALGAE

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DEDICATION

This thesis is dedicated to my family, particularly my brother and mother, for being there for me at all times, and under all conditions.

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ABSTRACT

Microalgae hold immense promise to help the world meet burgeoning energy and food needs. A major operational cost when commercially growing microalgae is the supply, storage, and delivery of carbon dioxide. To advance algal culture technique and improve cost effectiveness, this study sought to reduce CO_2 wastage without impeding algal growth. A carbon dioxide generation and delivery module (CGDM) was developed that delivers CO_2 generated on demand from bicarbonate solution. The system was successfully used to cultivate the green microalgae *Chlorella vulgaris* and *Dunaliella salina*. Using the CGDM, *C. vulgaris* culture reached a higher biomass concentration after a week of growth while using a small fraction of the CO_2 culture sparged with 0.4350% CO_2-enriched air required. As liquid carbonates can be easily obtained from waste CO_2, the approach described herein presents a sustainable solution to reduce production costs of algal culture while minimizing CO_2 emission to the environment.

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LIST OF ABBREVIATIONS AND TERMS

ANOVA: Analysis of Variance

BBM: Bold's Basal Medium

BCA: Bichinchoninic acid

CGDM: Carbon Dioxide Generation and Delivery Module

CO_2: Carbon dioxide

DMSO: Dimethyl sulfoxide

GFP: Green fluorescent protein

HCl: Hydrochloric acid

HCO_3^-: Bicarbonate (ion)

HPLC: High pressure liquid chromatography

LPM: Liters per minute

NADPH: Nicotinamide adenine dinucleotide phosphate

$NaHCO_3$: Sodium bicarbonate

PBR: Photobioreactor

PES: Polyethersulfone

PTFE: Polytetrafluoroethylene

ppm: Parts per million

PUFA: Polyunsaturated fatty acid

RP-HPLC: Reversed phase high pressure liquid chromatography

RuBisCO: Ribulose-1,5-bisphosphate carboxylase/oxygenase

Tukey's HSD test: Tukey's honest significant difference test

PREFACE

As the world grows more populous and economically developed, there is a correspondingly increased demand for energy, fuel, food, and other resources. Furthermore, with global warming and finite resources, there is a desire to find alternative resources that are renewable and ways to lower greenhouse gas concentration in the atmosphere, typically by fixing the carbon from carbon dioxide. Microalgae have the potential to help meet all of these challenges and opportunities. However, current methods of commercial-scale cultivation are too expensive to compete with existing technologies for similar products relying on non-sustainable sources. One major source of extra expense is the cost of carbon required for microalgal growth, encompassing carbon's supply, storage, and delivery to the site of

cultivation. Furthermore, under typical commercial-scale growth conditions using carbon dioxide gas as the carbon source, much of the CO_2 is lost due to outgassing into the atmosphere. Therefore, if carbon dioxide wastage is minimized or eliminated, considerable cost savings may be achieved in the production of microalgae, moving microalgae and its various downstream products and byproducts into more financially competitive positions. To this end, this study sought to ascertain whether a carbon dioxide delivery system could be created to minimize CO_2 loss while not impeding algal growth. A carbon dioxide generation and delivery system was developed and used to cultivate the freshwater, cell-walled green microalga, *Chlorella vulgaris*, as well as the saltwater, green microalga *Dunaliella salina*, which lacks a cell wall. The effect of gas delivery via diffusive mixing versus sparging with mixing was studied. To limit the amount of carbon used, several concentrations of carbon dioxide were tested. Additionally, this study investigated the effect of nitrogen source on algal growth and composition, specifically the use of nitrate and urea.

CHAPTER 1: INTRODUCTION

1.1 The Significance of Microalgae and Their Commercial Cultivation

There is increasing demand for consumable, carbon-based products obtained in a renewable and sustainable manner. For instance, biodiesel, green gasoline, and ethanol may serve as replacements for fossil fuels. Popular fertilizers and feedstocks also often contain petroleum-based ingredients. Not only is the use of non-renewable hydrocarbons unsustainable, but their processing and combustion releases massive quantities of greenhouse gases into the atmosphere: 31.2 gigatonnes carbon dioxide (CO_2) equivalent in 2012 (Tubiello et al., 2014). Meanwhile, plant sources of livestock and aquaculture feed may require the use of limited arable land, potentially leading to loss of various ecological habitats, biodiversity, and carbon sinks as new land is cleared to create farmland (Tubiello et al., 2014). Resource security is also frequently an issue of concern. For instance, in the state of Hawai‘i, all petroleum is imported as is all commercial livestock feed, as of 2013. (Ostrowski, 2013)

Microalgae can address issues such as these, and more. Green microalgae are photosynthetic unicellular eukaryotes that have garnered interest in the scientific and business communities for their potential to produce renewable fuels such as biodiesel and green gasoline, nutraceuticals and pharmacological products, industrial chemicals and materials such as glycerol or bioplastics, and as feed or fertilizer for livestock and plants (FAO, 2014; Gardner et al., 2013; Mendes, 1995; Pienkos & Darzins, 2009). For instance, several strains of algae have been found to produce the majority of their biomass as lipids which can be converted to biodiesel and other biofuels through downstream processing (Pienkos and Darzins, 2009). The remaining protein and carbohydrates can in turn function as ingredients in feedstock. Algae has the advantage over plants in that it can have drastically higher biomass/land ratio, or areal, productivity. Marine algae can also be grown on floating platforms in the ocean, further minimizing land use (Algae Systems, LLC). Additionally, microalgae have faster growth and photosynthesis rates than plants, especially those typically used for their lipids, proteins, or carbohydrates, with a theoretical maximal productivity of about 100g biomass m^{-2} day^{-1} for a typical green microalga (He et al., 2012; Pienkos and Darzins, 2009).

Current methods of algal cultivation include raceway ponds and photobioreactors (PBRs). Raceway ponds are more commonly used on a commercial scale, and typically

consist of an ellipsoidal containment pond to grow algae, with a paddle wheel used to keep the culture homogeneous and to mix the cells. The containment ponds are usually open to the atmosphere and their depths typically range from 0.25m to 0.4 meters in order to ensure sufficient solar radiation reaches the algae.

Photobioreactors are usually consist of enclosed, transparent vessels, often shaped to facilitate the ability of light to reach algal cells or the dissolution of carbon dioxide into the culture. Being a closed system, PBRs offer higher degrees of system control with significantly less water loss due to evaporation than their open pond counterpart (Slade & Bauen, 2013). PBRs are commonly used when the purity of a culture, or the minimization of contaminants is important. There exist hybrid open/closed systems that utilize PBRs where algal is primarily cultivated in a reservoir and then pumped into a photobioreactor where it receives carbon dioxide, other nutrients, or is filtered of wastes (Slade and Bauen, 2013).

These two modes of algal production have problems. While relatively inexpensive, raceway ponds' exposure to the environment leads to evaporation of water which must be replenished as well as contamination of the culture with bacteria, fungi, or other debris which might be undesirable for some applications, e.g. pharmaceuticals or nutraceuticals. The requirement that the ponds be shallow increases the area the ponds must cover, and algae cannot be economically grown year-round in environments that experience temperatures below freezing or low sunlight. PBRs are costlier than raceway ponds, including in terms of energy demand for running the more complex systems (Slade and Bauen, 2013).

One deficiency in both models is with regard to delivery of carbon dioxide. Microalgal growth is limited at dissolved carbon dioxide pressures of less than 0.1 kPa, considerably more than dissolved CO_2 in freshwater in equilibrium with atmospheric levels of carbon dioxide (Doucha et al., 2005). Comprising around half of the algal dry weight (Doucha et al., 2005), in many microalgal growth systems inorganic carbon is a limiting factor retarding optimal growth of the culture (Brune, D.E.; Goldman et al.). Furthermore, supplying this additional carbon is economically costly when conducted on an industrial scale (Doucha et al., 2005; Tapie and Bernard, 1988). The transfer and supply of carbon dioxide may comprise roughly one third to a half of the total cost to produce the algae, with the cost of carbon alone estimated at 8-27% of the daily production cost (Gris et al., 2014; Kumar et al., 2015; Li et al., 2013). One idea to get around this financial constraint is to locate the algal culture near to industrial sources that produce CO_2 as a waste product; however, in these

situations, issues such as land cost and light availability may come into play, making siting of a raceway pond or PBR farm unfeasible (Singh and Gu, 2010). Pumping flue gas to the algaculture sites would add further expense. Ideally, microalgae would be able to be grown on land or water that is readily available and not valuable for other purposes, and carbon could be supplied inexpensively. Therefore, there is demand to economize the use of CO_2 for cell growth, and to minimize loss of carbon to the atmosphere.

Contemporary methods of introducing CO_2 to algal mass culture in raceway ponds include the use of the following: sumps, a composite system employing a dual sump and airlift, an external carbonation column, covering the raceway pond to hold in CO_2, and, as mentioned above, hybrid systems using a photobioreactor for part of the cultivation and a raceway pond for the remainder (Kumar, 2015). While the existing methods of CO_2 delivery do seek to address CO_2 loss, there is still further refinement which must be done in order to improve or maximize CO_2 utilization. In raceway ponds, CO_2 is often wasted on account of the short residence time sparged gas bubbles have in the pond due to the pond's shallowness. When grown on nitrate media, the pH of the culture rises, affecting both the solubility of CO_2 into the media as well as the cell's ability to uptake inorganic carbon (Eriksen et al., 2007).

1.2 Microalgae

The number of microalgal species and strains known to exist are enormous, and a wide variety of algae are grown commercially depending on the product of interest, whether than be for biofuels, food, nutraceuticals, livestock feed, etc. (Milledge, 2011). Microalgae are typically unicellular microorganisms with a length measured on a micrometer scale which carry out photosynthesis converting carbon dioxide to organic molecules and producing oxygen gas as a byproduct (Andersen, 2013; Tebbani, 2014). These microorganisms are not monophyletic and consequently microalgae have great diversity regarding their genomes, phenotypes, biomolecular compositions, habitats, and other characteristics (Andersen, 2013).

Morphologically, microalgae can have varying appearances between species as well as due to different life stages of the organism or environmental conditions. For instance, microalgae can aggregate when exposed to stressors or flagellates can lose flagella when subjected to high shear stress. Common forms for microalgae include flagellate, coccoid, filamentous, sarcinoid, palmelloid or capsoid, and amoeboid (Andersen, 2013).

Microalgae can live in a wide variety of environments, including habitats that are hot, cold, acidic, alkaline, aqueous, terrestrial, etc. (Tebbani, 2014). Microalgae can grow at

differing levels of salinity, too, with freshwater, marine, and hypersaline species which thrive in these environs (Tebbani, 2014). Microalgae that live in both freshwater and saline locations possess unique characteristics worth exploiting for economic purposes.

The freshwater *Chlorella vulgaris* is one of the most well-studied and cultivated microalgae, grown for its nutritional profile and potential in biofuels research (Liu and Hu, 2013). A non-motile, ellipsoidal, unicellular microalga, *C. vulgaris* possesses a sturdy cell wall and is reliant on agitation of its growth medium to prevent its settling(Liu and Hu, 2013). This microalga reproduces asexually through the formation of 2-16 autospores during a reproductive cycle (Liu and Hu, 2013). *C. vulgaris* contains a cup-shaped chloroplast with a nucleus facing the concave region and ovoid mitochondria (Liu and Hu, 2013). It is capable of producing large quantities of protein and lipid as percentages of its biomass, and is tolerant of high concentrations of CO_2 as well as toxins such as nitrogen oxides and sulfur oxides which are commonly found in industrial waste gases (Liu and Hu, 2013; Tebbani, 2014). Furthermore, *C. vulgaris* can grow photoautotrophically, heterotrophically, and mixotrophically (Liu and Hu, 2013).

Dunaliella salina is a popular saltwater microalga grown commercially for β-carotene production (Milledge, 2011). *D. salina* lacks a cell wall, but is capable of withstanding a wide range of salinities and is a motile unicellular alga due to its possessing a pair of flagella (Borowitzka, 2013). The cells can have differing phenotypes based on growth conditions, such as changing in shape or color (Borowitzka, 2013). Like *C. vulgaris*, *D. salina* possesses a cup-shaped chloroplast, which is the site of β-carotene accumulation (Borowitzka, 2013). This alga is capable of sexual reproduction, which can involve a palmella stage and the production of isogametes, zygospores, and aplanospores (Borowitzka, 2013).

1.3 Carbon Sources for Algal Growth

Many microalgae are capable of growing mixotrophically, using carbon dioxide to carry out photosynthesis, as well as other inorganic or organic carbon substrates such as bicarbonate, glucose, fructose, glycerol, sucrose, acetate, etc. to fuel cell growth and division (Sharma et al., 2016). As it is relatively cheaply obtained from the atmosphere, and cannot be utilized by many non-photosynthetic organisms which may serve as contaminants in microalgal culture, carbon dioxide is frequently the primary carbon source in microalgal cultivation. However, there is considerable potential for the use of wastewater rich in carbon

compounds to grow algae, particularly algae grown for non-alimentary products (Wu et al., 2012).

1.4 Carbon Dioxide Delivery: Diffusion vs. Sparging

When carbon dioxide is used as the primary carbon source for algal culture, it is typical to sparge or bubble the gas into the culture medium in order to ensure good dissolution of the gas into the liquid. The carbon dioxide must first pass through the gas phase into the liquid phase of the medium through a gas-liquid interface in order to be uptaken by the microalgal cells (Carvalho and Malcata, 2001). Ideally, this transfer to the liquid phase should occur prior to CO_2 escaping out of the raceway pond or bioreactor into the atmosphere. However, if the carbon dioxide is being delivered in a more contained fashion, where loss outside the bioreactor system is minimal, it would be conceivable to introduce the CO_2 into the medium via diffusion, which would be more energy-efficient than sparging (Carvalho and Malcata, 2001).

1.5 Carbon Fixation Efficiency

The chief aim of this study was to maximize the efficiency of the uptake of carbon dioxide and its conversion to biomass. One way of measuring this is to calculate the carbon fixation efficiency, which seeks to quantify the amount of biomass obtained from a certain quantity of carbon. For microalgae, Li, et al. use the following formula to estimate the carbon fixation efficiency of carbon dioxide in a closed raceway pond, making the assumption that half the of the dry weight of the algae consists of carbon (Li et al., 2013):

$$\frac{0.5\,PV}{12 \times 60 \times 24 \times n_{CO2,supp}} \times 100\%$$

where 0.5 represents that fifty percent of the dry weight is carbon, P is the microalgal productivity in g L^{-1} day^{-1}, V is the gas flow rate of sparged carbon dioxide in L min^{-1} (LPM), 12 refers to the molecular weight of carbon, 60 is the number of minutes in an hour, and 24 is the number of hours in a day.

1.6 Nitrogen Sources for Algal Growth

Depending on the species and strain, microalgae can meet their requirement for nitrogen by consuming nitrogen in the form of several substrates (Flynn and Butler, Ian, 1986). In the scientific and industrial fields, it is typical to supply nitrogen in the form of nitrate, ammonium, or urea for microalgal cultivation (Lourenço et al., 2002). Typically,

nitrate's metabolism results in a raising of pH, ultimately to cytotoxic levels, by the following reaction (Eriksen et al., 2007):

$$CO_2 + H_2O + 0.15NO_3^- + 0.15H^+ \rightarrow CH_2O(NH_3)_{0.15} + 1.3O_2$$

in which protons are consumed, leading to an elevation in pH. Ammonium, on the contrary, can result in a drop in pH due to the following net biochemical reaction:

$$CO_2 + H_2O + 0.15NH_4^+ \rightarrow CH_2O(NH_3)_{0.15} + 1.0O_2 + 0.15H^+$$

which concludes with an increase in proton concentration and a corresponding drop in pH (Eriksen et al., 2007; Scherholz and Curtis, 2013). In contrast to nitrate and ammonium, urea's metabolism, involving two reactions (Hodson et al., 1975):

$$CO(NH_2)_2 + ATP + HCO_3^- \xrightarrow{urea\ carboxylase} C_2H_3N_2O_3^- + ADP + P_i + H^+$$

$$C_2H_3N_2O_3^- + 3H_2O + H^+ \xrightarrow{allophanate\ lyase} 2NH_4^+ + 2HCO_3^-$$

does not result in a net pH change. Therefore, if one seeks to alter or maintain the pH of an algal culture, one has the option of doing so by manipulation of the nitrogen source in the growth medium. For instance, for acidophilic algae, providing nitrogen as ammonium might be preferable, as a low pH will be maintained, potentially limiting bacterial contamination. Similarly, nitrate could be used for alkophilic algae. A combination of ammonium and nitrate could even be used to encourage algal growth if desired (Scherholz and Curtis, 2013).

1.7 Summarization and Focus of the Present Study

Based on review of the literature and commercial microalgal ventures, it is apparent that notable cost-savings can be obtained by reducing carbon dioxide wastage, and by increasing the carbon fixation efficiency. Additionally, further savings and increased biomass yield might be achieved by replacing sparging of gas with diffusive dissolution and by altering the nitrogen source in the medium to prevent an extreme rise or fall in pH.

1.7.1 Objectives

The research described in this thesis aimed to culture high-quality algae economically by developing a new carbon dioxide gas generation and delivery module to produce CO_2 on demand from the reaction of a bicarbonate solution with an acid, and to deliver the carbon gas to the algal culture in such a way as to minimize carbon dioxide wastage and maximize the carbon fixation efficiency. This system was designed to be able to be used in both

freshwater and hypersaline environments without appreciable corrosion so that it could be used in the cultivation of both freshwater and saltwater algae.

To this end, factors such as whether or not sparging of the gas was necessary and what level of CO_2 dosage was appropriate were investigated. Also, this study sought to determine whether the substitution of urea into microalgal media typically prepared with sodium nitrate would be acceptable for microalgal culture, and whether this substitution would result in a stable pH of the culture.

1.7.2 Hypotheses

It was hypothesized that such a carbon dioxide generation and delivery module (CGDM) could be constructed and used to cultivate the freshwater green microalga, *C. vulgaris*, and the saltwater green microalga, *D. salina*.

It was further hypothesized that some degree of sparging would be required for optimal cell growth, with intermittent sparging being adequate for this purpose. This was on account of both the need for a relatively rapid dissolution of carbon dioxide into the liquid phase but also reduced concern for CO_2 leaking into the atmosphere as the CO_2 gas was largely contained by the CGDM (Figure 1).

An additional hypothesis was that a relatively low concentration of carbon dioxide, a partial pressure below 0.5%, would be sufficient to achieve optimal growth without imposing a limit on carbon supply. The reason for this hypothesis was that CO_2 could be generated and delivered to the cells in a rapid enough fashion that low levels of CO_2 per dose were sufficient and that this dosing in small aliquots would reduce the amount of gas leakage out of the CGDM-photobioreactor (CGDM-PBR) system.

It was also hypothesized that the use of urea in lieu of nitrate would result in a stable pH of the algal culture, and that this stable pH might allow for a longer exponential phase of cell growth and division, and therefore a larger final biomass at the end of the test cultivation runs.

CHAPTER 2: MATERIALS AND METHODS

2.1 Microalgal Cultivation

2.1.1 Chlorella vulgaris

Chlorella vulgaris UTEX 395 was obtained from the UTEX Culture Collection of Algae. The culture was maintained in Bold's Basal Medium (BBM) (Bischoff, 1963) in Erlenmeyer flasks with air sparging on an orbital shaker operating at 90 rpm, at 21.5°C, and under lighting from cool white or natural yellow fluorescent lightbulbs delivering 30-50 μmol m^{-2} s^{-1} to the culture continuously. Subculture was conducted every 10 days. For tests using nitrate as the nitrogen source, unmodified BBM was used. For the reactor runs using urea as the nitrogen source, the microalgae were cultivated in a modified BBM substituting nitrate for urea in a 1:1 molar ratio and replacing cobaltous nitrate with an equimolar amount of cobalt chloride. Algae for these runs were maintained in this same modified medium. Cultures grown on urea were pH-adjusted to 8.0 with HCl and/or NaOH prior to treatments F-I. Exponential-phase culture were used to inoculate the PBR. Prior to inoculation, algae were centrifuged at 3,214×g for 3 minutes and resuspended in 500 mL of freshly-prepared BBM to an absorbance at 550 nm of 0.20 ± 0.05. The system was lit continuously (24 hr day^{-1}) by a toroidal fluorescent bulb delivering approximately 30 μmol m^{-2} s^{-1} to the PBR. The temperature of the culture was 25 ± 1°C.

2.1.2 Dunaliella salina UTEX 1644

D. salina UTEX 1644 was maintained by reserving a 100 mL portion of a culture cultivated in the CGDM with approximately 300 μmol m^{-2} s^{-1} of lighting by four toroidal fluorescent bulbs, mixing using the impeller at 40 rpm. This reserved culture was kept in a 500 mL baffled Erlenmeyer flask under similar shaking and lighting conditions as described for *C. vulgaris*, and was maintained for no more than seven days under these conditions prior to inoculation of the CGDM-PBR. A modified Pick's Medium (Li et al., 2003) was used to cultivate the algae with sodium nitrate replaced by 10 mM urea. Urea was added to autoclaved medium as an aqueous solution of less than 10 mL volume by the use of a 0.2 μm-pore hydrophilic polyethersulfone (PES) syringe filter (VWR) and syringe immediately after inoculation of the medium with algae. Prior to addition to the PBR, algae from the reserve was centrifuged at 3,214×g for 3 minutes and resuspended in in 500 mL of freshly prepared modified Pick's Medium to an absorbance at 550 nm of 0.2 ± 0.05.

2.2 Construction and Setup of the Carbon Dioxide Generation and Delivery System

2.2.1 Basic setup of the CGDM

A bench-scale stirred-tank photobioreactor (PBR) with the integrated CO_2 generation and delivery module (CGDM) was designed and constructed as shown schematically in Figure 1. The CGDM consisted of three interconnected chambers: one for housing an IR-based CO_2 gas sensor (Vernier) to monitor pCO_2, another for CO_2 generation by combining $NaHCO_3$ and HCl solutions, and a third chamber that was partially submerged in the algal culture to facilitate CO_2 transfer to the medium while reducing CO_2 loss (Figure 1). The reactor chambers were constructed using Plexiglas acrylic sheets and tubes.

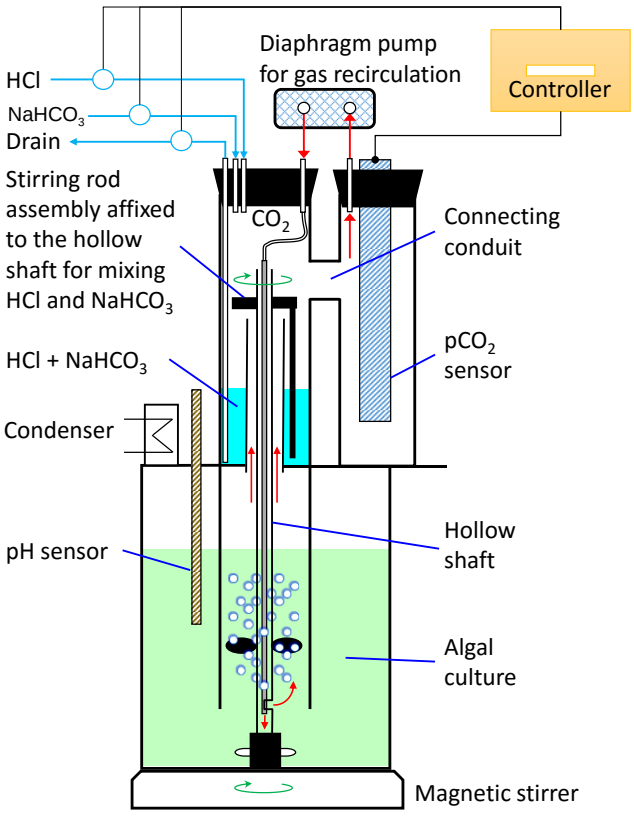

Figure 1: Schematic of Carbon Dioxide Generation and Delivery Module and photobioreactor (CGDM-PBR) system. Red arrows indicate flow of air throughout the system, circulated by the micro diaphragm pump.

Each time the pCO_2 dropped below a set value, a liquid pump was activated by a controller to drain spent CO_2 generation solution for 30 seconds prior to the delivery of new $NaHCO_3$ and HCl solutions into the CO_2-generation chamber until the pCO_2 registered a set upper value (e.g. 0.25- 0.50% CO_2). The CO_2 controller using an Arduino Uno microcontroller board was programmed using Arduino software, and was designed to

measure CO_2 partial pressure in the gas-generation chamber every second, and output the data via serial interface every 10 minutes.

Using a micro diaphragm gas pump (KNF, Model NMP 830 KNDC B) that ran constantly, the CO_2 gas was drawn from the gas-generation chamber and sparged into the algal culture through a stationary tube inserted into a rotating hollow shaft with a slit cut out for releasing the gas into the culture at a pre-determined gas flow rate (Figure 1). This micro diaphragm pump was used for all treatments except for *D. salina* grown at 0.15 LPM, which used a smaller gas-sampling micro diaphragm pump capable of lower aeration rates (KNF, Model NMP 09 M). The gas bubbles released from the slit were dispersed through the aid of an axial-flow impeller installed on the hollow shaft. The diaphragm pump served to circulate the gas throughout the entire module. A stirring-paddle was affixed to the rotating hollow shaft to aid mixing of the HCl and $NaHCO_3$ solutions, promoting a more rapid CO_2 generation and release, and therefore a quicker detection of a rise in pCO_2 by the CO_2 sensor, minimizing pCO_2 overshoot. A magnetic stir bar was connected to the bottom of the hollow shaft. The rotation of the entire shaft/impeller assembly was driven by a magnetic stirrer station (Barnstead Therolyne).

C. vulgaris and *D. salina* were grown in the reactor vessel into which the CGDM was inserted. Ports were constructed to accommodate a pH probe, and for sampling plus venting of gas through a condenser.

2.2.2 Arduino Programming

An Arduino Uno microcontroller board was connected to the following: a CO_2 sensor, a liquid pump (LeadFluid) capable of pumping from two vessels simultaneously, and a liquid pump (SimplyPumps) for drainage of CO_2 generation solution. The programming of the Arduino was by Arduino software using a Sony VAIO laptop. The program contained options for modifying the drainage time, the upper and lower pCO_2 values, the delay time between drainage and the initiation of the pump, and how often the pCO_2 was read—which was set at one second for all treatments.

2.2.3 CGDM configuration to determine the benefits/detriments of diffusion vs. sparging

For experiments to determine whether strong algal culture with a high carbon fixation efficiency could be achieved by use of convective diffusion alone (without sparging), the diaphragm pump was not employed. All such treatments used nitrate as the nitrogen source, and such tests were done solely on *C. vulgaris* cultures (Table 1).

2.2.4 CGDM configuration to determine an adequate dosage of carbon dioxide

Several pCO_2 concentrations were tested to try to determine the lowest pCO_2 dosage necessary to achieve optimal cell growth. For *C. vulgaris*, first atmospheric CO_2 and then constant sparging at 0.4 LPM of 1% CO_2-enriched air was used. When the CGDM was employed, a pCO_2 setting of 0.5-1.0% for the lower and upper values was used, before trying a setting of 0.25-0.5%. Gas flow rates were set to 1.7 LPM.

For experiments using *D. salina*, the settings were set at 0.25-0.5% pCO_2, but the sparging rates were altered to test growth at 1.00 LPM, 0.50 LPM, and 0.15 LPM.

2.2.5 CGDM configuration to determine the benefits/detriments of using sodium nitrate vs. urea as the nitrogen source

To determine if urea-based medium resulted in equal or superior growth to nitrate, a pCO_2 setting of 0.50-1.00% was used, with a sparging rate of 1.7 LPM.

Table 1: Various setup configurations of the Carbon Dioxide Generation and Delivery Module for growth of *C. vulgaris*. Treatment is the iteration used, and as referred to in the text. Nitrogen source refers to either nitrate supplied as 2.94 mM $NaNO_3$ or urea at a 2.94 mM concentration. Unless otherwise stated, air flow was set at 1.7 LPM.

Treatment	Description	Nitrogen Source	Gas Delivery
A	Carbon supplied by atmospheric CO_2.	Nitrate	Vortexed Diffusion
B	1% CO_2 sparged into reactor at 0.4 liters per minute.	Nitrate	Constant Sparging
C	CO_2 generated from the reaction of bicarbonate and acid. Lower pCO_2 limit: 0.50%; Upper pCO_2 limit: 1.00%	Nitrate	Vortexed Diffusion
D	CO_2 generated from the reaction of bicarbonate and acid. Lower pCO_2 limit: 0.50%; Upper pCO_2 limit: 1.00%	Nitrate	Intermittent Sparging
E	CO_2 generated from the reaction of bicarbonate and acid. Lower pCO_2 limit: 0.50%, then 0.25%; Upper pCO_2 limit: 1.00%, then 0.50%	Urea	Intermittent/Constant Sparging
F	CO_2 generated from the reaction of bicarbonate and acid. Lower pCO_2 limit: 0.25%; Upper pCO_2 limit: 0.50%	Urea	Constant Sparging
G	Constant sparging of atmospheric air	Urea	Constant Sparging
H	0.435% CO_2-enriched air sparged into reactor	Urea	Constant Sparging
I	CO_2 generated from the reaction of bicarbonate and acid. Lower pCO_2 limit: 0.25%; Upper pCO_2 limit:0.50% (Replicate 2)	Urea	Constant Sparging
J	*D. salina*. Lower pCO_2 limit: 0.25%; Upper pCO_2 limit 0.50% 0.15 LPM	Urea	Constant Sparging
K	*D. salina*. Lower pCO_2 limit: 0.25%; Upper pCO_2 limit 0.50% 0.15 LPM	Urea	Constant Sparging
L	*D. salina*. Lower pCO_2 limit: 0.25%; Upper pCO_2 limit 0.50% 0.15 LPM	Urea	Constant Sparging

2.2.6 CGDM configuration for the cultivation of D. salina

For the cultivation of *D. salina*, only the effect of sparging rate was tested. Rates of 1.0, 0.5, and 0.15 LPM were used. For tests using 0.15 LPM, a small diaphragm pump capable of 0.15 LPM was used as the original diaphragm pump had a minimum pump rate of 0.5 LPM. Lighting was raised by the addition of three toroidal fluorescent bulbs around the PBR, elevating light levels to 300 μmol m^{-2} s^{-1}. The axial flow impeller speed was reduced to 40 rpm. Modified Pick's medium with urea in lieu of nitrate as described in subsection 2.1.2 was used. Cultivation conditions were otherwise the same as for *C. vulgaris*.

2.3 Measuring Microalgal Growth

2.3.1 Optical density at 550, 750, and 680 nm

The optical density, or absorbance, of algal cultures were measured using a Shimadzu UV-160U spectrophotometer at one or more of the following wavelengths: 550 nm, 750 nm, or 680 nm. Samples consisted of one milliliter of algal culture or diluted algal culture which was micropipetted into a plastic Plastibrand cuvette with a path length of one centimeter. Tests on samples were performed 3-4 times per day. For treatments F-I, four aliquots of one milliliter algal culture were drawn, diluted as needed to keep the absorbance reading below 0.5, and their absorbances read. Other treatments either followed the procedure of F-I or consisted of measuring a single sample 3-4 times.

2.3.2 Hemocytometry

Hemocytometry was performed using a Hausser Scientific Ultra Plane hemocytometer slide and an Olympus BX60 optical microscope. Cells within eight randomly chosen squares were counted for each sample and used to calculate the cell concentration according to the manufacturer's specifications. Two individual samples were drawn from the algal culture and diluted as needed for all treatments.

2.3.3 Dry Weight

Dry weight was obtained by centrifuging an algal sample at 75,000 rpm and either drying it for three days in a 60°C oven or for forty-eight hours in a Labconco freeze dryer.

Estimates in this text for dry weight within the seven-day cultivation periods are based on a dry weight calibration curve for *C. vulgaris* and *D. salina* using absorbances at 550 nm and 750 nm. For treatments F-I, calculations made using dry weight are based upon dry weight estimates obtained from absorbances at 550 nm only. Four measurements were taken and calculated.

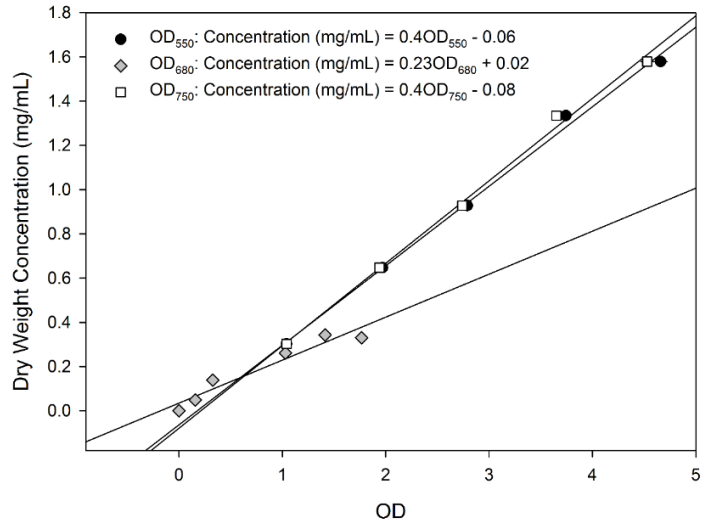

Figure 2: Standard curves for *C. vulgaris* measured at 550 nm, 680 nm, and 750 nm absorbance.

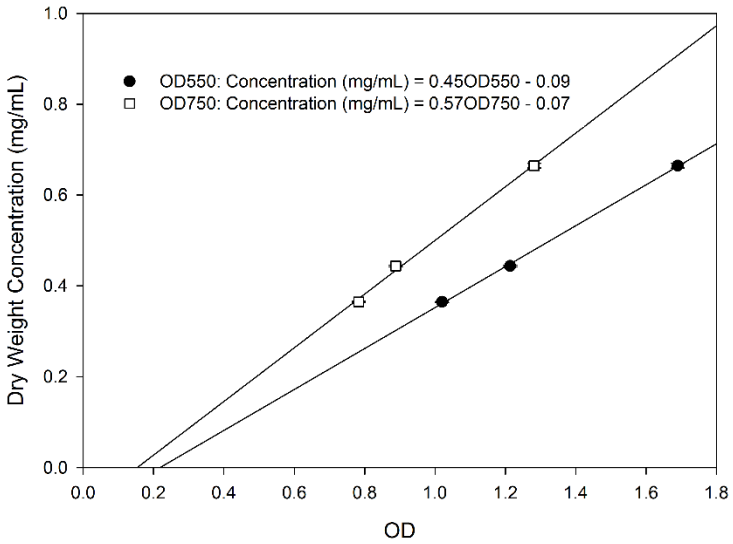

Figure 3: Standard curves for *D. salina* measured at 550 nm and 750 nm absorbance.

2.4 pH

The pH was measured by a Vernier pH sensor and LoggerLite software. pH was recorded daily manually. Additional a timecourse pH profile was generated by the LoggerLite software, recording the pH every ten minutes for the course of one week.

2.5 Carbon Usage

2.5.1 Sodium bicarbonate demand

The 0.272 M sodium bicarbonate used in the following experiments was contained in a 100 mL graduated cylinder, sealed to prevent large losses of water due to evaporation. The amount of bicarbonate solution pumped to produce CO_2 was recorded daily. The moles of bicarbonate, CO_2, and carbon were calculated based on multiplying the volume consumed by the molarity. Equal volumes of 0.272 M $NaHCO_3$ and 0.274 M HCl were combined to produce CO_2 and a NaCl solution of approximately pH 4.0 to ensure that more than 99% of the carbon would be converted to CO_2. The volume of generation solution was typically less than or equal to two milliliters, and thus the amount of carbon remaining in solution was taken to be negligible.

2.5.2 pCO_2 concentrations

The partial pressure of CO_2 in the airspace of the CGDM-bioreactor system was recorded every ten minutes for one week using a Vernier CO_2 sensor and LoggerLite software in parts per million (ppm). The Vernier CO_2 sensor was set to measure in the 0-100,000 ppm range.

2.5.3 Carbon fixation efficiency

The carbon fixation efficiencies were calculated by using the averaged dry weight concentration estimates in g L^{-1} multiplied by the volume in liters divided by the moles of carbon based on the bicarbonate usage. This value was multiplied by 100% to get a percentage.

2.6 k_La

The gas transfer performance in the PBR with or without the cylindrical baffle partition (Figure 1) was compared by measuring the volumetric oxygen mass transfer coefficients in the PBR using the dynamic gassing-in method with a dissolved oxygen electrode (Mettler-Toledo Ingold, Billerica, MA) inserted in place of the pH electrode (Fig. 1). k_La measurements were done in 500 mL of either deionized water or algal culture grown for seven days. Nitrogen gas was used to displace dissolved oxygen from water or culture

until zero percent oxygen was recorded after which oxygen was sparged into the reactor at a constant rate. The k_La for CO_2 was calculated by multiplying the k_La values for oxygen by 91% in accordance with the findings of a previous study (Kordač and Linek, 2008).

2.7 Nitrate

Nitrate was measured daily using a HACH Cat. No. TNT 835 assay kit following the supplied protocol. A standard curve was made using sodium nitrate. For each measurement, an algal sample was drawn from the PBR and diluted tenfold. One and a half milliliters of diluted sample were pipetted into a 1.7 mL microfuge tube and centrifuged at 7,500 rpm for five minutes. One mL of supernatant was pipetted into the assay reagent vial containing dimethylphenol. Then 200 µL of Reagent A containing sulfuric acid was added to the vial, and the vial capped and contents mixed by inversion of the vials several times. The sample was incubated for 15 minutes. The sample was then pipetted into a quartz cuvette with a path length of one centimeter and the absorbance read four times at 345 nm using a spectrophotometer (Shimadzu UV160-U).

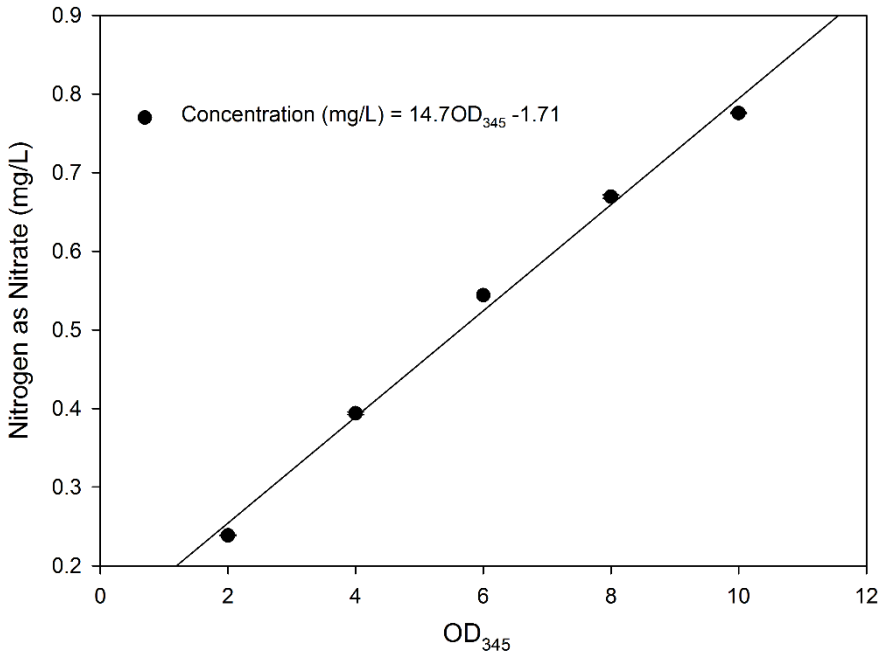

Figure 4: Standard curve for nitrate based on sodium nitrate.

2.8 Urea and Ammonia

Urea and ammonia were measured daily using a Megazyme urea/ammonia (Rapid) assay kit (Cat. No. K-URAMR) following the vendor's protocol. Three algal samples were

drawn and diluted as needed prior to centrifugation at 7,500 rpm for five minutes. For each sample, in a plastic Plastibrand cuvette with a path length of one centimeter, one milliliter of deionized water was pipetted along with 50 µL of supernatant. Next, 150 µL of supplied pH 8.0 buffer solution was added, along with 100 µL NADPH solution. The samples were then mixed by pipetting repeatedly and incubated for two minutes. The absorbance at 340 nm was then read and recorded using a spectrophotometer (Shimadzu UV160-U). After reading the absorbance, glutamate dehydrogenase (GlDH) enzymatic mixture was shaken to homogenize the mixture and 10 µL added to the samples. The samples were then incubated for five minutes, and the absorbances read and recorded as before. The urease mixture was shaken gently to homogenize and then 25 µL of urease was pipetted into each sample and mixed by pipetting. Samples were incubated for five minutes and then the absorbances read and recorded as before. If the absorbances were not stable, new readings and recordings were taken at one minute intervals until a constant reading was obtained.

Relevant chemical reactions involved in the assay are as follows (Megazyme):

$$urea + H_2O \xrightarrow{urease} 2NH_3 + CO_2$$

$$NH_3 + H_2O \rightarrow NH_4^+ + OH^-$$

$$2-oxoglutarate + NADPH + NH_4^+ \xrightarrow{GlDH} L-glutamic\ acid + NADP^+ + H_2O$$

2.9 Carbohydrates

2.9.1 Total carbohydrates

Total carbohydrates were measured for *C. vulgaris* using the Phenol-Sulfuric Acid Method. In brief, one milliliter algal sample was drawn and centrifuged at 7,000 rpm for 5 minutes, the supernatant removed, and the pellet resuspended in deionized water. This sample was then pipetted into a glass bacterial culture tube with an additional one milliliter of deionized water. Next, 50 µL of 80% phenol was added to the diluted sample, capped, and well-mixed by vortexing for 30 seconds. Following this, five milliliters of sulfuric acid was pipetted by serological pipette into the culture tube, capped, and the final mixture mixed by vortexing as before. The tubes were allowed to incubate until room temperature, approximately 40 minutes, before vortexing, pipetting 700 µL into a quartz cuvette with a one centimeter path length, and reading the absorbance of the solution at 490 nm in a spectrophotometer (Shimadzu UV-160U). A standard curve was generated with glucose from 0-50 µg mL^{-1} following the same procedure.

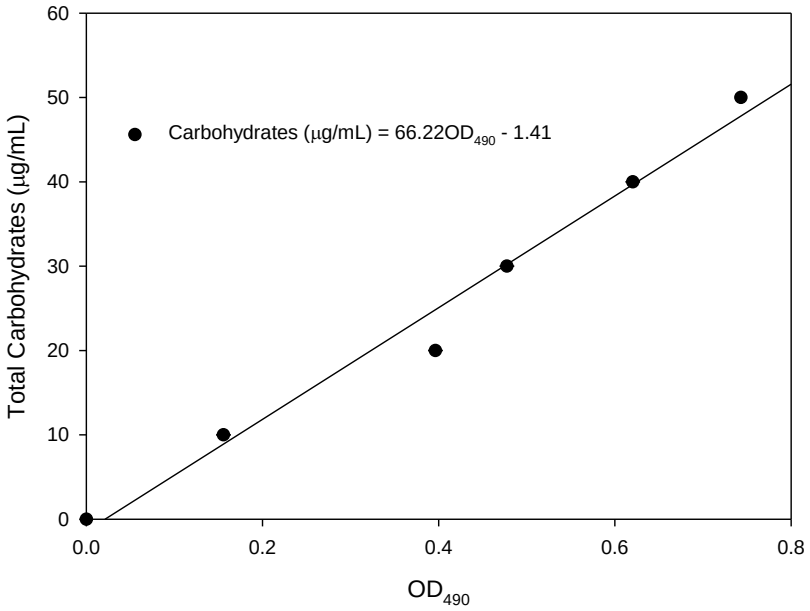

Figure 5: Standard curve for total carbohydrates based on glucose.

2.9.2 Starch

Starch was measured using an EnzyChrom Starch Assay Kit (BioAssay Systems Cat. No. E2ST-100) following the manufacturer's instructions. A standard curve was made simultaneously with starch quantification of the algal samples, using supplied materials. In brief, lyophilized *C. vulgaris* was frozen in liquid nitrogen and ground to a powder with mortar and pestle. Three ~7 mg samples were added individually to 1.7 mL microfuge tubes (VWR). Free glucose and extraneous polysaccharides were removed by washing with one milliliter 95% ethanol, warmed to 60°C for five minutes in a dry bath incubator (Fisher Scientific), with vortexing after three minutes after which samples were returned to the incubator. Samples were then centrifuged at 10,000×*g* for two minutes. The supernatant was removed by pipetting, and the washing process repeated twice more. After removal of the ethanol, one milliliter deionized water was pipetted. The samples were then incubated at 95°C for five minutes in the dry incubator, prior to centrifugation as before. The supernatant containing soluble starch was removed into a separate 1.7 mL microfuge tube. 200 μL of dimethyl sulfoxide (DMSO) was added to the pellet containing resistant starch, and the sample heated at 95°C for five minutes, and centrifuged as described previously. The resistant starch sample was diluted 100 fold before assaying.

Ten microliters of standard and algal starch samples were added to a 96-well microplate. Working reagent was prepared by mixing Assay Buffer 1, Enzyme A, Enzyme B, and Dye Reagent in a 90:1:1:1 ratio. 90 µL of working reagent was added to each sample and the solutions mixed by tapping. Blanks were made in like fashion using deionized water instead of algal sample. Samples were incubated at room temperature for one hour, and the fluorescence read at 570 nm. Starch was calculated by taking the difference between the fluorescence of the sample and that of the blank divided by the slope of the standard curve.

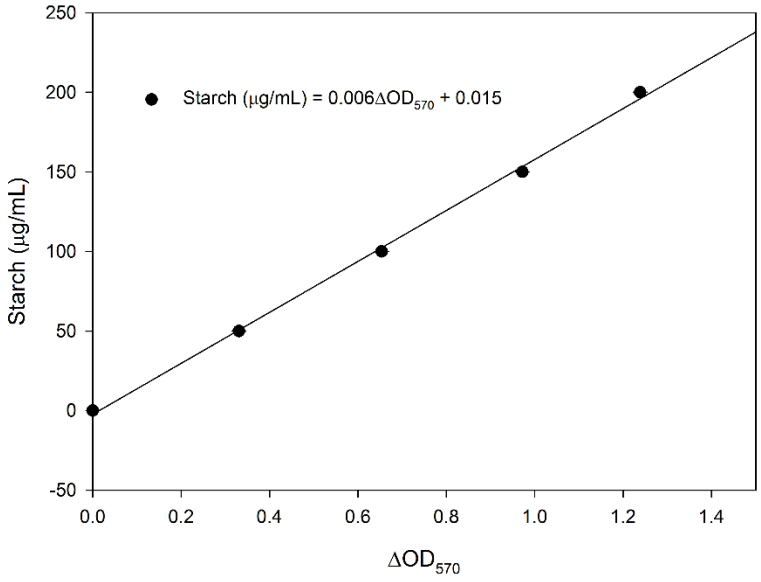

Figure 6: Standard curve for starch.

2.10 Lipids

2.10.1 Folch Method

Lipids were extracted using the Folch Method (Folch et al., 1957). In summary, three algal samples of approximately five grams were frozen in liquid nitrogen and ground by mortar and pestle. A 2:1 chloroform-methanol mixture was added, with a volume twenty times that of the weight of the algal sample, making the assumption that the chloroform-methanol mixture had a density of approximately one gram per milliliter. The mixture was mixed by shaking and incubated for one hour, after which the homogenate was centrifuged at 10,000 rpm. 0.9% NaCl was used to wash the sample, with one fifth the volume of the homogenate used for this purpose. The mixture was then centrifuged at 2,000 rpm and the phases allowed to separate. The upper phase was removed by pipetting, and the lower phase

containing lipids was allowed to dry in a fume hood prior to weighing in an analytical balance (Mettler Toledo AB54).

2.10.2 Nile Red

A calibration curve for the rough quantification of lipids was made using Nile Red dye and olive oil as the standard. 3.14 μL of 0.318 ug mL^{-1} Nile Red was added to 995 μL of 50% DMSO to make a working solution. 500 uL of working solution was added to 500 μL of sample or standards in 1.7 mL microfuge tubes and the samples vortexed. Samples consisted of algal culture were drawn from the PBR. The samples were incubated in the dark for 10 minutes. 700 μL samples were drawn and deposited into a quartz cuvette with a path length of one centimeter, and then fluorescence was read at an excitation wavelength of 530 nm and an emission wavelength of 570 nm.

2.11 Protein

2.11.1 Lowry Assay

Protein content was determined based on the procedure detailed in Slocombe et al. (Slocombe, 2013). Briefly, *C. vulgaris* was centrifuged at 7,500 rpm and the pellet lyophilized, then frozen with liquid nitrogen and mechanically pulverized with a mortar and pestle. Approximately five milligrams of algal biomass per sample, three in total, were mixed with 24% trichloroacetic acid and incubated for 15 minutes at 95°C, after which the samples were allowed to cool before diluting with deionized water, centrifuged and the supernatant discarded. The precipitate was resuspended in Lowry reagent D and incubated overnight at 65°C prior to centrifugation. Solubilized protein in the supernatant was then measured using the Lowry assay. Lowry assays were performed on each of the three samples twice.

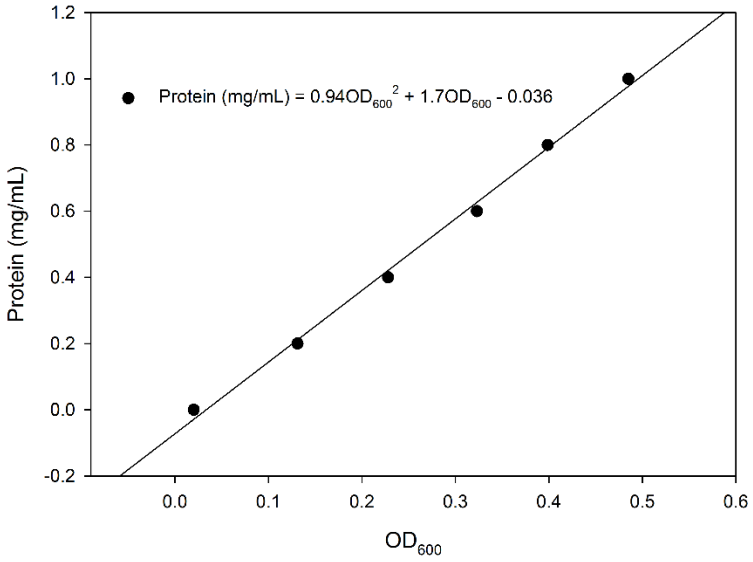

Figure 7: Lowry Assay protein calibration curve based on bovine serum albumin.

2.11.2 Smith Assay

Protein was also measured using the Smith Assay (Olson and Markwell, 2007). A standard curve was made using bovine serum albumin. In summary, lyophilized algal samples were frozen in liquid nitrogen and ground by mortar and pestle. Samples approximately 50 mg in weight were measured out into a 1.7 mL microfuge tube. One milliliter of 4% copper sulfate pentahydrate was combined with 49 mL bicinchoninic acid (BCA). 50 µL of this solution was added to the algal sample and mixed by repeated pipetting. One milliliter of the copper sulfate-BCA solution was added to each sample and mixed by vortexing. The samples were then incubated at 37°C for 30 minutes, and then cooled to room temperature. Samples were then pipetted into a quartz cuvette with a path length of one centimeter and the optical density read at 562 nm in a spectrophotometer (Shimadzu UV160-U).

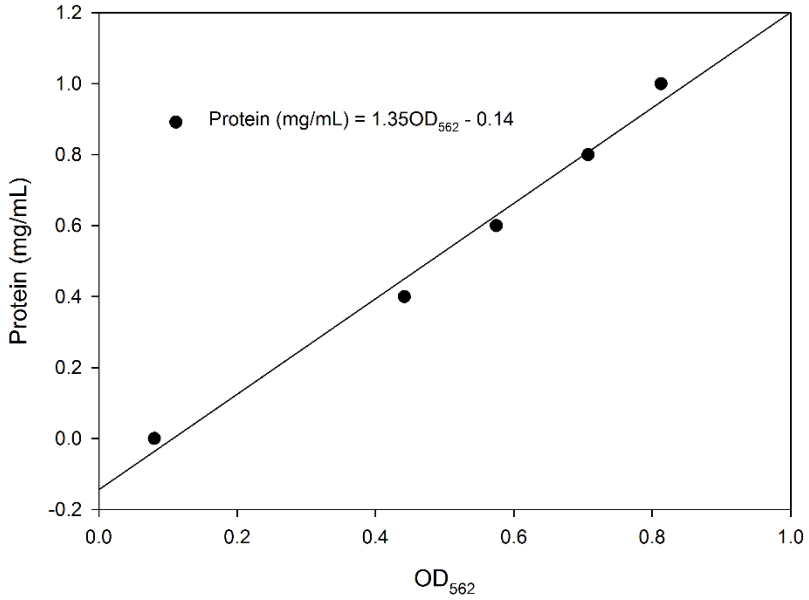

Figure 8: Standard curve for protein using the Smith (BCA) Assay based on bovine serum albumin.

2.12 Pigments

2.12.1 Chlorophyll

Chlorophyll content was estimated by centrifuging one milliliter algal samples, diluted as needed, at 7,500×g for five minutes and resuspending the cell pellet in one milliliter methanol and incubating at 65°C for a half hour prior to centrifuging again at 13,500×g for two minutes then measuring the absorbance of the supernatants at 664 nm and 647 nm and using the following formulae (Lichtenthaler and Wellburn, 1983):

$$Chlorophyll\ a\ \left(\frac{ug}{mL}\right) = DF(12.7 * A664nm - 2.69 * A647nm)$$

$$Chlorophyll\ b\ \left(\frac{\mu g}{mL}\right) = DF(22.9\ A647nm - 4.68\ A664nm)$$

$$Total\ Chlorophyll\ \left(\frac{\mu g}{mL}\right) = DF(20.3\ A647nm + 8.02\ A664nm)$$

where *DF* is the dilution factor, *A647nm* is the absorbance at 647 nm, and *A664nm* is the absorbance at 664 nm. Four measurements were taken on a daily basis.

2.12.2 Total carotenoids

For *D. salina*, pigments were extracted in methanol as for *C. vulgaris* and then measured at absorbances of 653 nm, 666 nm, and 470 nm, after which the following formulae were applied to estimate chlorophyll and carotenoid content (Lichtenthaler and Wellburn, 1983):

$$C_a\ \left(\frac{\mu g}{mL}\right) = 15.65OD_{666} - 7.34OD_{653}$$

$$C_b\ \left(\frac{\mu g}{mL}\right) = 27.05OD_{653} - 11.21OD_{666}$$

$$C_{x+c}\ \left(\frac{\mu g}{mL}\right) = \frac{1000OD_{470} - 2.86C_a - 129.2C_b}{245}$$

where C_a is chlorophyll a, C_b is chlorophyll b, and C_{x+c} is total carotenoids. Four measurements were taken on a daily basis.

2.12.3 β-carotene and lutein

Lutein and β-carotene were purchased as supplements. Lutein standard was prepared from six milligram lutein softgel capsules (CVS) and β-carotene from 25,000 IU softgel capsules (NatureMade). One capsule of each was perforated and submerged in 200 mL of 50% methanol, 50% methylene chloride, both HPLC grade. Samples were then filtered through a PTFE syringe filter prior to reversed phase high pressure liquid chromatography (RP-HPLC) analysis.

For the construction of standard curves, β-carotene extracts of 150, 375, and 750 μg mL^{-1} were used. For lutein, extracts of six, 15, and 30 μg mL^{-1} were used (Figure 9). Frozen samples of *D. salina* were thawed, and approximately one gram wet weight algae was weighed out into 25 mL Pyrex bottles. Extraction of carotenoids followed the method of Hu, et al. (Hu et al., 2008). In brief, algae were added to 25 mL Pyrex bottle and 25 mL of an extraction solution of hexane, 95% ethanol, and acetone in a 2:1:1 ratio was added. The gelatin softgel casing had to be perforated to release lutein from the capsule, and the sample was incubated for 24 hours in the dark. 1.25 mL of 40% methanolic KOH was then added, and the mixture shaken and incubated in the dark overnight. The extract was then filtered through a 0.2 μm-pore filter and washed twice with water. The carotenoid-containing phase was separated using a serological pipette and allowed to evaporate in a fume hood. Extracts were then dissolved in 10 mL of a methanol-methylene chloride solution (1:1, v/v) and filtered through PTFE syringe filters prior to RP-HPLC analysis.

RP-HPLC analysis generally followed the procedure of Hu, et al. (Hu et al., 2008), with the following modifications: a five micrometer, 150 x 4.6 mm, Luna C18 column (Phenomenex) was used with the methanol/acetonitrile/water: methylene chloride ratio being 85:15. Absorbance readings were at 448-450 nm. The RP-HPLC using a Shimadzu HPLC system was run at one milliliter per minute until after the lutein peak was observed and

typically for approximately 15 minutes. Chromatography was performed and chromatograms created using EZStart software.

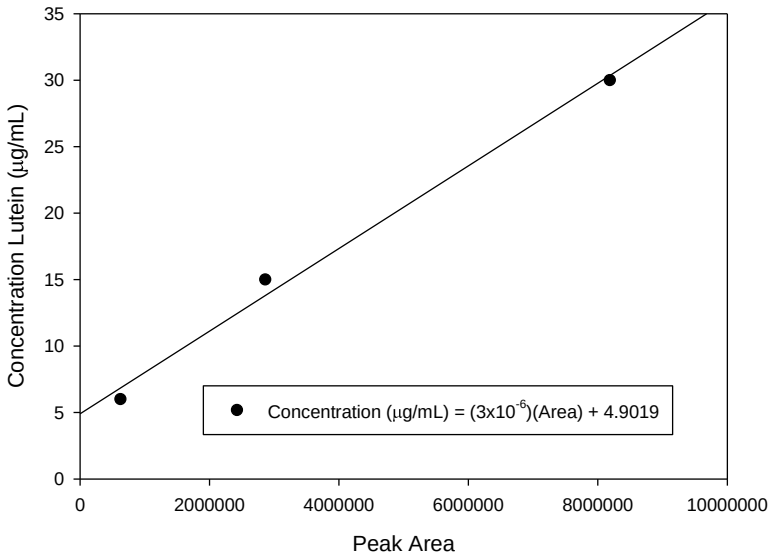

Figure 9: Standard curve for lutein measured via RP-HPLC.

2.13 Microalgae Storage and Preparation for Assays

After each test cultivation run, the ~500 mL of algal culture was centrifuged at 75,00 rpm for three minutes in a pre-weighed 50 mL centrifuge tube and the supernatant decanted prior to filling the same tube with ~50 mL of culture and centrifuging again under the same conditions. This was repeated until the entire algal culture for the test run was pelleted in a single 50 mL centrifuge tube. The algal samples were then frozen -80°C in a freezer.

For *C. vulgaris* tests of protein, lipid, and starch, frozen samples were then lyophilized in a Labconco freeze dryer for forty-eight hours.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Operation of the CGDM

The CGDM performed as designed. There was a slight overshoot in CO_2 generation as there was a delay between the CO_2 generation in the reaction chamber and the transport of that generated carbon dioxide to the CO_2 sensor chamber. For example, at a CGDM setting of 0.25-0.5%, where the CGDM would be triggered when the pCO_2 measured 0.25% and would generate CO_2 until a reading of 0.50% was recorded—and the theoretical average pCO_2 would be 0.375%—the pCO_2 rose almost to 0.6% (6,000 ppm) with each dosing, as illustrated in Figure 10C, and the average pCO_2 for the duration of the cultivation was 0.4350%. The inclusion of a rotating paddle to mechanically mix the HCl and $NaHCO_3$ solutions and drive out from solution CO_2 gas bubbles formed, as well as continuous circulation of gas in the headspace of the CGDM-PBR system from the sparger, ameliorated this issue with pCO_2 overshooting.

Basing the triggering of the CGDM on an arbitrary low pCO_2 value as opposed to triggering periodically on a time basis had the advantage of generating and delivering CO_2 to the system as needed. For instance, early in the culture, soon after inoculation, the cell concentration was low, and therefore demand for CO_2 was low and the CGDM only needed to be triggered infrequently (Figure 10C). As the algal density increased, demand for CO_2 correspondingly increased, and the CGDM was triggered more frequently in order to supply the needed carbon dioxide. Had the CGDM been set to run at uniform time intervals, there would likely have been excess CO_2 generated in the early phase of cultivation, or insufficient CO_2 produced towards the end of the week-long growth period. Although there was a slight overshooting of CO_2 generation, with the pCO_2 often reaching around 0.6% instead of the upper pCO_2 bound of 0.5%, the CGDM maintained the pCO_2 of the system within a tight range.

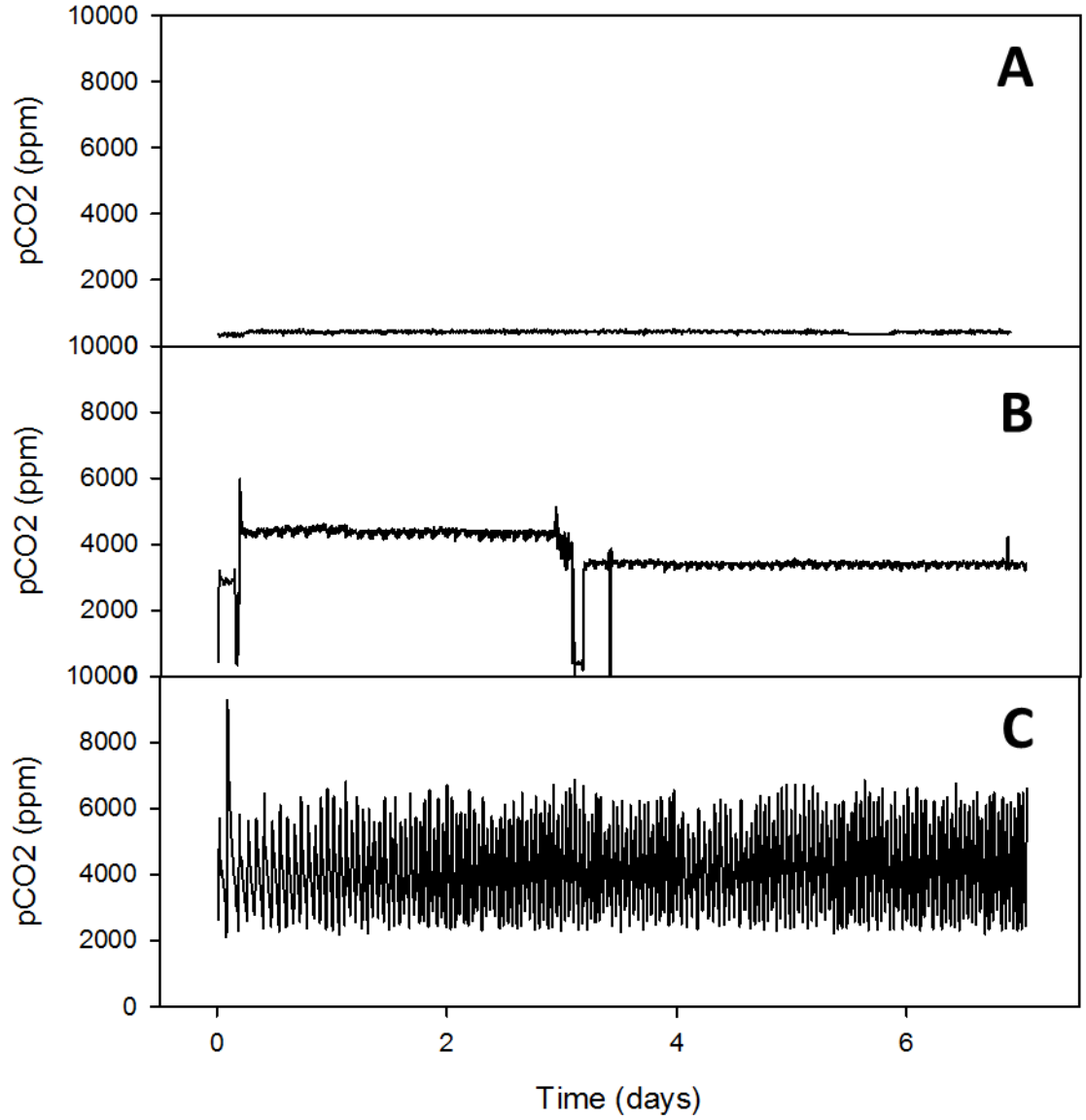

Figure 10: pCO_2 recorded every ten minutes for the course of a week-long algal culture. (A) Treatment G, a negative control sparging air into a *C. vulgaris* culture at 1.7 LPM. (B) Treatment H, a positive control sparging 0.4350% CO_2-enriched air into a culture at the same rate as in A. On the third day of cultivation, the CO_2 tank ran out of carbon dioxide and had to be replaced; this is reflected the drop in pCO_2 recorded in this time frame. (C) Treatment I, a test run of the CGDM set at a lower pCO_2 limit of 0.25% and an upper limit of 0.50%, also growing *C. vulgaris*. The time interval between triggerings of the CGDM decreased as the cell culture density rose.

3.2 Growth of Microalgae

For all treatments except for *C. vulgaris* grown on nitrate under conditions of diffusion of atmospheric CO_2 and for *D. salina* grown at a sparging rate of 1.0 LPM there was noticeable algal growth (Figures 11, 12).

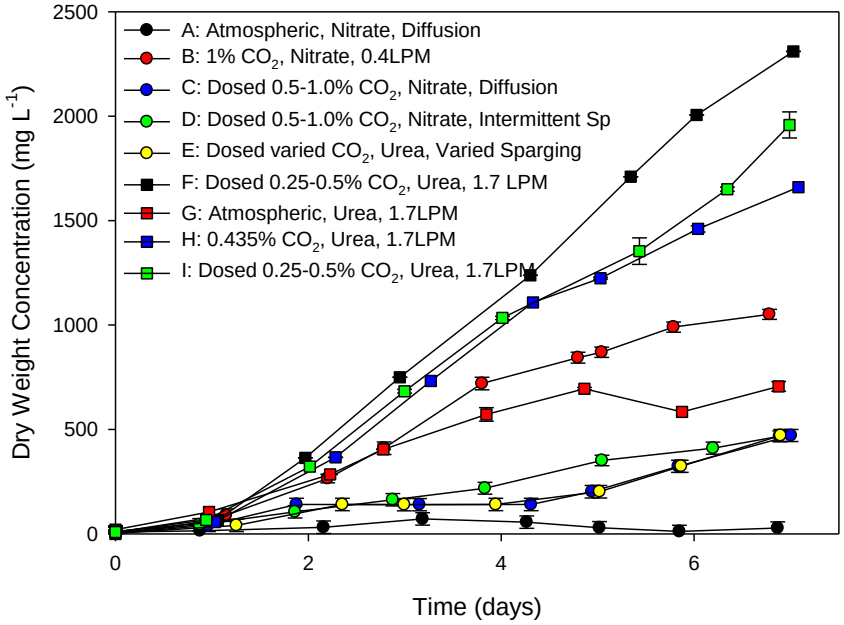

Figure 11: Dry weight as concentration for *C. vulgaris* cultures grown under different CGDM setups. Treatments F and I were under the same growth conditions, except treatment F was carried out for approximately 13 days.

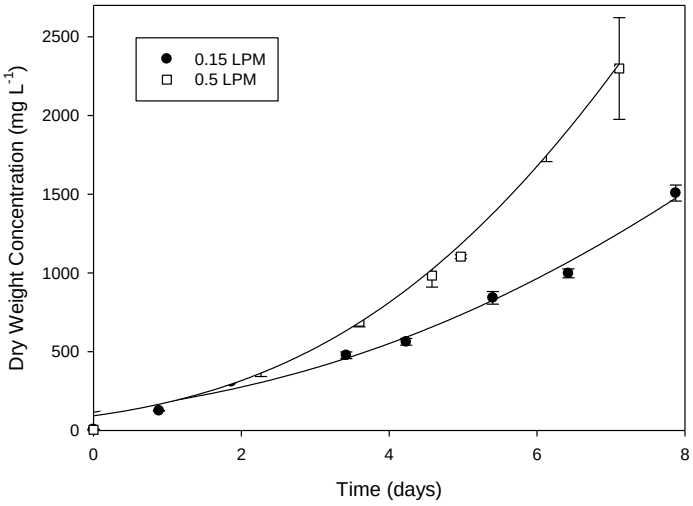

Figure 12: Growth of *Dunaliella salina* at gas sparging rates of 0.15 and 0.5 LPM. (Growth at 1.0 LPM was negative, and is not shown.)

The low growth for algae grown using the diffusion of atmospheric CO_2 is likely on account of a limitation of carbon supply combined with inadequate agitation of the medium to remove wastes from the algae, such as the removal of dissolved oxygen, and to promote

the even distribution of nutrients and light, etc. (Grobbelaar, 1994). For *Dunaliella* grown at a sparging rate of 1.0 LPM, it is unclear whether the sparging rate was so violent that shear stress caused the cells to grow poorly (the cells showed signs of stress such as the loss of their flagella and chlorophyll, and underwent carotenogenesis) or if this poor growth was due to cells not being sufficiently photo-acclimated to 300 μmol m^{-2} s^{-1} of lighting prior to inoculation of the PBR.

3.2 Sparging is Necessary for Robust Growth

The initial test of the CGDM, referred to as 'A,' was performed using air for CO_2 supply, nitrate as the nitrogen source, and diffusion with mixing by the magnetic stirring bar and axial flow impeller for gas transfer. As can be seen from Figure 11 and 13, treatment A, intended to be a negative control, resulted in poor growth. As an early stage positive control, treatment B used air enriched with an additional 1% CO_2 which was sparged into the CGDM-PBR system at a rate of 0.4 LPM; again, nitrate was the nitrogen source. As expected, this achieved high growth but at the expense of carbon fixation efficiency. Treatment C was the first test of the CGDM's dosing mechanism and programming. The system was configured to generate and deliver CO_2 when the pCO_2 reading measured 5,000 ppm, or 0.50%, and was set to shut off when the recorded pCO_2 read 10000 ppm, or 1.00%. In order to save on expenses, we sought to observe whether high growth could be accomplished with no sparging, but rather with gas delivery solely through convective diffusion in a mixed medium. Unfortunately, as can be seen in Figures 11 and 13, treatment C did not deliver high biomass. However, it was fairly efficient in terms of carbon fixation.

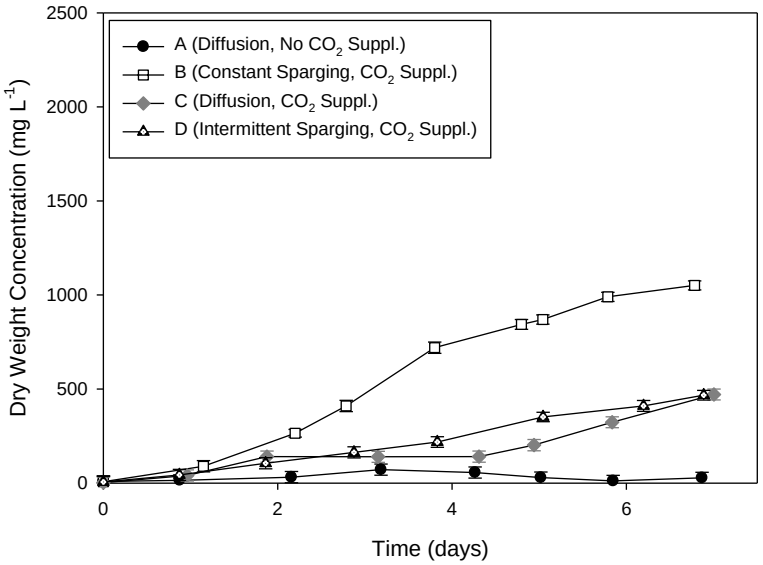

Figure 13: Cell growth of *C. vulgaris* under treatments A-D. Diffusion refers to diffusion of carbon dioxide by molecular equilibrium and vortexing. CO_2 Suppl. Refers to supplementation with carbon dioxide in excess of atmospheric levels. Best growth was observed under conditions of constant sparging with CO_2 supplementation.

The results of treatments A, B, and C lead to the conclusion that aeration of the culture via sparging might enhance algal growth, given treatment B's much higher biomass than treatment C's, although both supplied high levels of CO_2. Treatment D used dosed CO_2 using the CGDM system as before, with sparging set to run for three minutes upon the start of CO_2 generation. This intermittent sparging led to almost the same level of biomass as C, suggesting that intermittent sparging of a duration of only a few minutes was still inadequate for good growth. Consequently, it was concluded that either an extended period of intermittent sparging or continuous sparging was required for strong cell growth, and it was decided that future testing would consist of cultures sparged continuously.

3.3 Urea is an Acceptable Substitute for Nitrate for *C. vulgaris* and *D. salina*

Bold's Basal medium (BBM) typically uses nitrate as the main source of nitrogen for the algal culture. At 2.94 mM, nitrate was largely depleted by seven days in treatments C and D, and had been consumed by the third day for treatment B (Figure 15). Therefore, at the standard concentration of nitrate in BBM, nitrogen is limiting over the course of a week-long run using the CGDM, especially with sparging of CO_2-enriched air such that the culture is not carbon limited. Furthermore, nitrate's metabolism results in a raising of pH, ultimately to

cytotoxic levels (Eriksen et al., 2007). In Figure 17, one observes a strong rise in pH for treatments C and D. Treatment A, which registered negligible algal growth, still resulted in an elevation of pH. Treatment B shows an apparent stable pH, but this is due to sparged CO_2 acting as a pH buffer for the system. The two stable levels shown in Figure 17 for treatment B are the result of the CO_2 being depleted during the course of the run. While not being sparged with CO_2, the culture pH rose, and then stabilized as a new tank of CO_2 gas was added to the system. (Figure 10B). As urea is of similar cost to sodium nitrate per mole, and provides twice the amount of nitrogen at equimolar concentrations, it is a valid alternative to nitrate for the many algae that can metabolize it, such as *C. vulgaris* and *D. salina*. Urea's metabolism, involving two reactions converting urea first to allophanate and then allophanate to ammonium ion does not result in a net pH change (Hodson et al., 1975).

It was surmised that by maintaining culture pH of near neutral would lead to optimal growth, and for the reasons described above, urea was substituted for sodium nitrate in the formulation of BBM, also replacing cobaltous nitrate with cobalt chloride to remove the trace amount of nitrate contributed by that molecule. As can be seen from Figure 14, treatments D and E resulted in similar dry weight concentrations are the end of seven days while being run under similar conditions with an exception being the nitrogen source. However, treatment E was still in the exponential phase, while treatment D had reached senescence as described later, suggesting that had the cultivation run been extended the biomass for treatment D would have continued to increase.

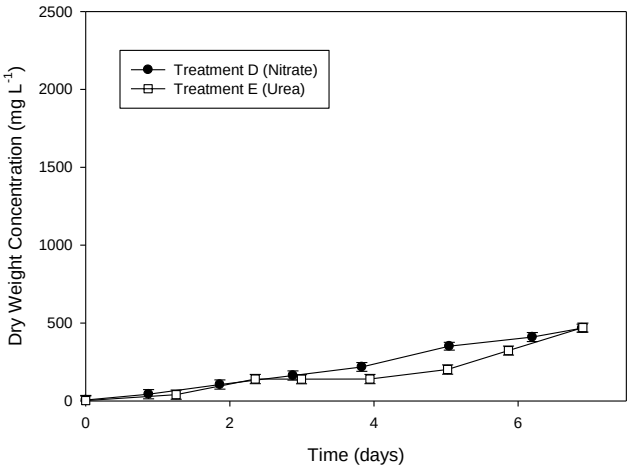

Figure 14: Cell growth for *C. vulgaris* under treatments D and E using nitrate and urea as the nitrogen source, respectively. Cell concentration and growth was similar for algae grown under both nitrogen sources. However, treatment D was reaching senescence as of the seventh day whereas treatment E was still in its exponential phase.

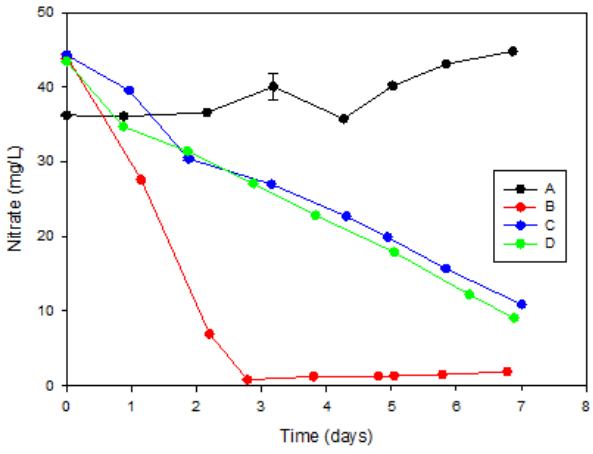

Figure 15: Nitrate consumption by the culture over the course of a seven day period under treatments A, B, C, and D.

Figure 16 demonstrates that urea was also consumed by seven days, particularly for the test run of the CGDM (treatment I) and for the corresponding positive control where air enriched with 0.435% CO_2 was sparged into the CGDM-PBR system. However, even in the corresponding negative control, treatment G, where unenriched air was sparged into the CGDM-PBR, urea was largely used up. Thus, it appears that even with twice the available

nitrogen, nitrogen is still a limiting nutrient of the CGDM-PBR under these conditions, particularly for the dosed test run of the CGDM.

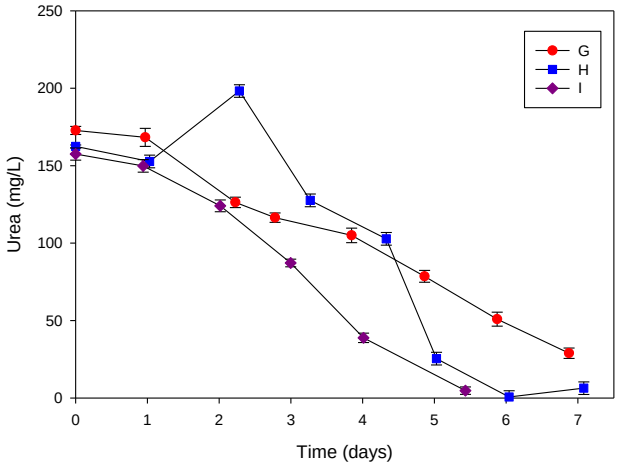

Figure 16: Urea consumption by *C. vulgaris* over time for treatments G, H, and I.

As mentioned, the pH of the culture rose for cultures grown on nitrate (Figure 17), the exception being treatment B, where air enriched with 1% CO_2 led to an acidification of the medium by CO_2 that counteracted the proton abstraction by nitrate metabolism. In contrast, for cultures using urea as the nitrogen source, treatments E-I, pH remained largely stable. Treatment E displays an anomalous trend, with a reduction of pH later in the experiment when the CO_2 dosage settings was lowered from 0.5-1.0% CO_2 to 0.25-0.5% CO_2. One idea for this is that the urea was hydrolysed into ammonia without enzymatic assistance but simply by being in water for an extended period of time, and the ammonia was metabolized by the algae. Ammonia's metabolism follows a reaction which results in a rise in proton concentration and a corresponding drop in pH (Eriksen et al., 2007).

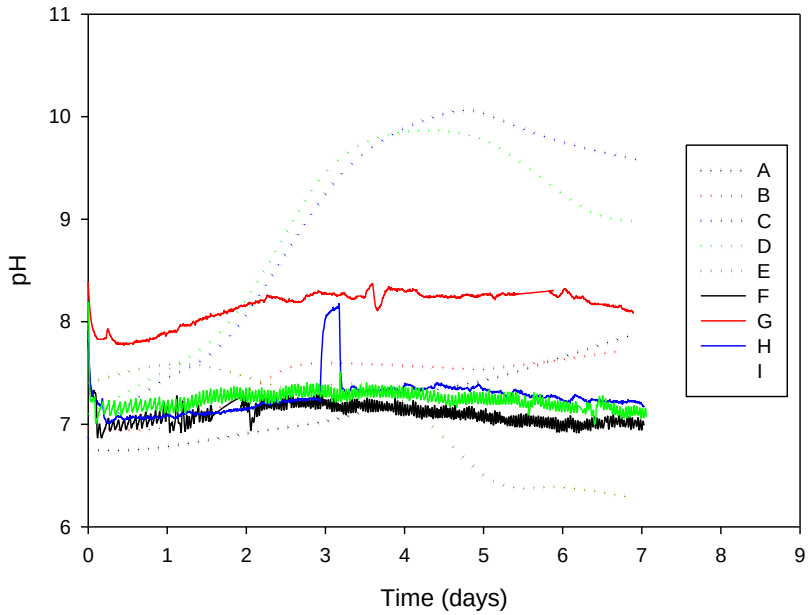

Figure 17: Culture pH over time for algae cultivated under differing CGDM treatments, A-I. For nitrate using cultures that grew well (C, D), the pH rose to almost 10 prior to senescence. For urea using cultures, the pH remained largely static.

3.4 Low Levels of CO_2 Dosing is Sufficient for Strong Growth

The data obtained from treatment E led us to conclude that urea would be an acceptable, even preferable, substitute for nitrate. After seven days, the dry weight concentration for culture grown on urea was similar to culture using nitrate, with the urea growth curve indicating the culture was still in exponential phase even if nitrogen was depleted or nearing depletion (Figure 14). For treatment D, where culture reached pH levels at which growth is halted (Goldman et al.), cell culture was in a senescence phase on the seventh day of cultivation. Furthermore, carbon dioxide was not being used as efficiently as it might. Typical pCO_2 levels of CO_2 enrichment for sparged gas may range from 0.5-1.0%, however these concentrations often serve the additional role of buffering the culture's pH as well as providing carbon to the system (Carvalho and Malcata, 2001; Pegallapati and Nirmalakhandan, 2012; Singh and Singh, 2014). With urea replacing nitrate, and the concomitant stable pH of the algal culture, these levels of CO_2 were deemed unnecessary.

With these concerns in mind, it was then decided to set up the CGDM-PBR using a dosing range of 0.25-0.5% CO_2, with constant sparging at 1.7 LPM, and to use urea as the nitrogen source for the cultivation of *C. vulgaris*. Treatments F and I (Figure 11, 18) demonstrate strong growth under these conditions—superior, in fact, to the biomass achieved

under constant sparging at 0.4 LPM with 1% CO_2 enriched air in nitrate BBM (treatment B) and a new positive control using constant sparging at 1.7 LPM with 0.435% CO_2-enriched air in urea BBM (treatment H). Therefore, it was demonstrated that a comparatively lower concentration of CO_2 could be used to achieve high cell growth with high efficiency.

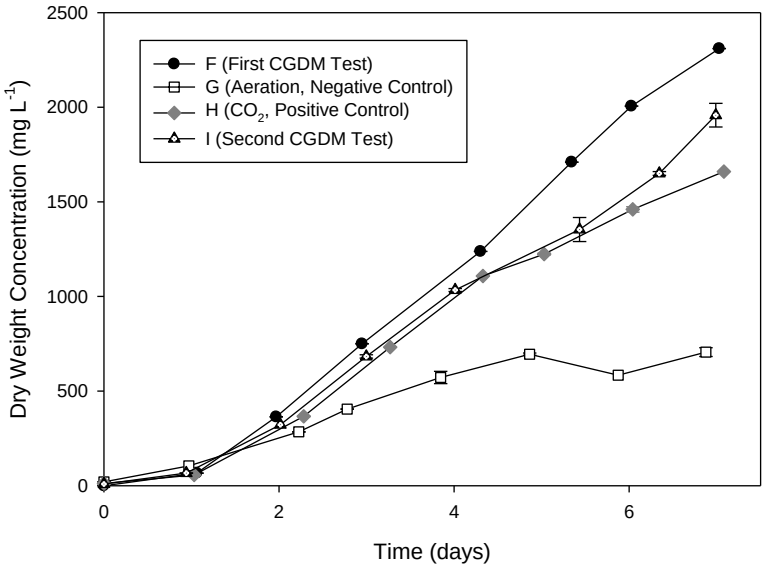

Figure 18: Cell growth of *C. vulgaris* for treatments F-I. F: the first test of the CGDM using urea, constant sparging at 1.7 LPM, and a pCO_2 range of 0.25-0.5%. G: a negative control using urea, constant sparging at 1.7 LPM, and atmospheric air. H: a positive control using urea, constant sparging at 1.7 LPM, and 0.435% CO_2-enriched air. I: the second test of the CGDM under the same conditions as F.

As mentioned previously, this setup did use up urea prior to the end of the cultivation period and maintained a stable pH near neutral. With more urea supplied, it is probable that a higher biomass might have been realized.

3.5 Oxygen Toxicity

High oxygen levels in the algal culture can lead to impaired cellular growth due to photo-oxidative damage and inhibition of photosynthesis. Under a high partial pressure of oxygen the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) functions increasingly as an oxygenase; ultimately this results in glycine condensation and the release of previously-fixed CO_2 (Kumar, 2015). The activity of ribulose-1,5-bisphosphate carboxylase is also competitively inhibited by excess oxygen (Ogawa et al., 1980). The dissolved oxygen level in the *C. vulgaris* culture of treatment I at the end of the run on day

seven was measured at 124.9 ± 2.5% air saturation (equivalent to 10.3 ± 0.2 mg L^{-1} in water at 25°C).

Dissolved oxygen concentrations in raceway ponds typically can reach as high as 35-30 mg L^{-1} without causing growth inhibition (Kumar, 2015). The calculated dissolved oxygen in a seven-day old *C. vulgaris* culture grown using the CGDM was 10 ± 1 mg L^{-1}. Outgassing of dissolved oxygen from the continuously illuminated CGDM culture via diffusion to the atmospheric headspace was sufficient to prevent accumulation of oxygen to an inhibitory level, and to allow the culture to achieve active growth.

3.6 Biomolecular Composition of Algae Grown under a CGDM Regimen

While the goal was to produce a high biomass of algae while minimizing carbon usage, we were also interested in the composition of the cells grown under the conditions of the CGDM, and how they compared to more traditional methods of growth—sparging constantly with CO_2-enriched air or air without CO_2 enrichment. For reference, Figure 18 illustrates algal growth for treatments F-I, as reflected by the dry weight concentration. As a reminder, Treatments F and I were test runs of the CGDM set at a lower pCO_2 limit of 0.25% and an upper limit of 0.5%, Treatment G was a negative control consisting of the aeration of air from the atmosphere, and Treatment H was a positive control consisting of constant sparging of air containing 4,350 ppm CO_2. All four treatments were sparged at 1.7 LPM and involved the cultivation of *C. vulgaris*.

A summary of amounts of chosen biomolecules of interest for *C. vulgaris* (treatments F-I), and *D. salina* (D1-D3), are compiled in Table 2.

Table 2: Values for dry weight concentration and several molecules of interest after seven days* for treatments F-I cultivating *C. vulgaris*, and for two treatments cultivating *D. salina*. D1: *D. salina* grown at a sparging rate of 0.15 LPM; D2: *D. salina* grown at a sparging rate of 0.5 LPM. Error is in standard deviations. For dry weight and chlorophyll, n =4. For protein, starch, and lipids, n = 3.

Treatment	Dry Weight (g/L)	Starch (% Dry Weight)	Lipid (% Dry Weight)	Protein (% Dry Weight)	Chlorophyll (% Dry Weight)
F*	2.310 ± 0.000	5.79 ± 1.36	26.8 ± 2.0	22.7 ± 1.0	1.98 ± 0.01
G	0.706 ± 0.023	5.16 ± 1.49	27.3 ± 0.5	34.7 ± 2.9	2.78 ± 0.09
H	1.660 ± 0.000	4.86 ± 0.97	30.6 ± 2.6	35.3 ± 2.8	1.86 ± 0.00
I	1.958 ± 0.062	6.48 ± 0.54	24.0 ± 1.9	37.1 ± 2.4	1.83 ± 0.06
D1	1.508 ± 0.051	----------	----------	----------	3.02 ± 0.10
D2	2.299 ± 0.323	----------	----------	----------	0.81 ± 0.13

**Treatment F was run for approximately 13 days.*

3.6.1 Carbohydrates

Among other downstream products, microalgae are cultivated for their carbohydrates, which can serve as a food source for aquaculture or livestock, in some instances even for human consumption. For instance, a sizeable percentage of microalgal carbohydrate exists as starch or glycogen, which is readily digested by animals. Different algae produce different forms of starch, with green algae largely producing amylopectin, while red algae produce floridean starch, and cyanobacteria, glycogen (Markou et al., 2012). Microalgae contain other polysaccharides such as carrageenan, a common thickening agent food additive, as well as other sulfated polysaccharides like ulvan and fucoidan, which show potential for the development of new pharmaceuticals and functional foods (Vaz et al., 2016). The carbohydrate content of microalgae is also valuable for the synthesis of biofuels, such as bioethanol, biohydrogen, and biobutanol (Markou et al., 2012). Microalgae are particularly desirable for biofuel productions which require microbial fermentation as they lack hemicellulose and lignin, which are not easily fermented (Markou et al., 2012). Therefore, the carbohydrate content of *C. vulgaris* was measured to ascertain what effect, if any, use of the CGDM had on carbohydrate and starch concentration.

Prior to treatment A, there was interest in seeing the levels of several biomolecules in *C. vulgaris* using an earlier version of the CGDM-PBR system. To measure carbohydrates, the phenol-sulfuric acid method was used, which is a way to quantify all carbohydrates in an organic substance. The results of a five day cultivation period are depicted in Figure 19.

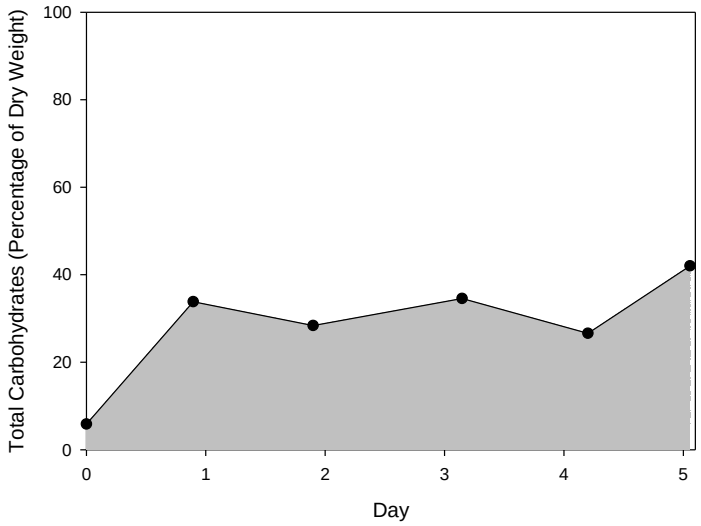

Figure 19: Total carbohydrates in *C. vulgaris*. Given as a percentage of dry weight, by day of cultivation.

As can be seen, the percentage of algal dry weight that was carbohydrate remained relatively steady at around a third of the total dry weight. The initial day of cultivation registered a rather low percent of carbohydrate, and this is likely due to error based on the measurement being performed on an algal sample of a very low concentration.

While useful to quantify the total sugars in an organic substance, the phenol-sulfuric acid method measures as carbohydrates molecules such as cellulose or other polysaccharides that are not easily digested as food or convertible to renewable energy. Therefore, it was determined that for experimentations using the more complete version of the CGDM, a starch assay would be employed to measure a carbohydrate that is desirable as a food source for animals and microbes and can be converted easily to renewable energy sources such as ethanol.

The measured starch for treatments F-I are illustrated in Figure 20. Both soluble and resistant starch were measured. There was no major difference between treatments, with around five percent of the total dry weight consisting of starch, a considerably smaller value that the estimated percentage that was any form of carbohydrate. This demonstrated that use of the CGDM did not adversely affect starch levels compared to more traditional methods of algaculture.

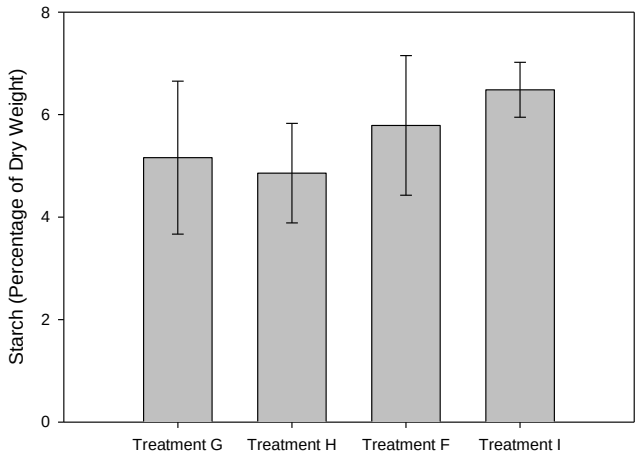

Figure 20: Starch content for treatments F-I. The percentage of dry weight that was starch (soluble and resistant). There was no significant difference ($p < 0.01$) between treatments.

3.6.2 Lipids

Microalgae also produce lipids, sometimes as a very large percentage of their dry weight. *C. vulgaris* can hold as much as 60% of its dry weight as lipid under low nitrogen and phosphorous conditions (Zuñiga et al., 2016). This attribute makes microalgae attractive as a source for the synthesis of biofuels such as biodiesel and biogasoline (Pienkos and Darzins, 2009). Microalgal lipids can also serve as a source for molecules such as the polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and γ-linolenic acid (GLA) which are sought-after by the health and food industries directly for human consumption, or to produce more nutritious fish and livestock containing these PUFAs (Vaz et al., 2016). Thus, the determination of lipid content was measured, again to learn if microalgal growth by CGDM affected, adversely or beneficially, lipid concentration in the cells.

There was initially interest in studying the accumulation of lipid as the algal runs progressed, on a daily basis as for measurements of cell growth, nitrogen consumption, pH, chlorophyll, etc. However, the Folch Method requires the use of samples that are large in terms of the amount of algae that can be obtained from the CGDM-PBR system used in this thesis, particularly in the first few days of cultivation. For this purpose, a Nile Red-based estimation of lipid content of *C. vulgaris* algal cells was attempted. The resulting estimates are shown in Figure 21.

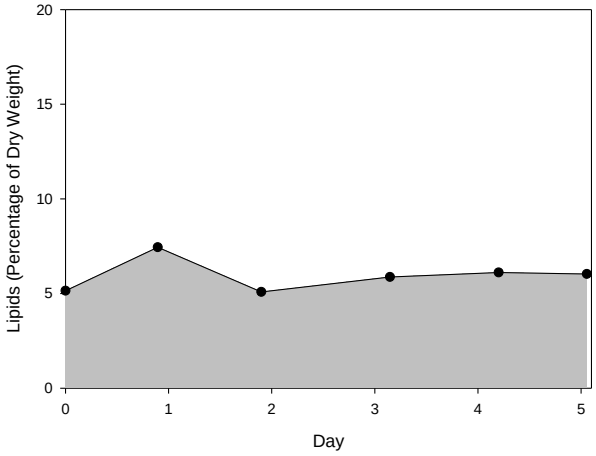

Figure 21: Timecourse estimates for lipids as a percentage of *C. vulgaris* dry weight over five days based on Nile Red fluorescence.

The estimates gave a consistent value suggesting that around five percent of the total dry weight was lipid. These values, while possible, were lower than that typical for *C. vulgaris*, even when the culture is not nitrogen-limited.

At the end of treatments F-I, lipid measurements were made using the more accurate Folch Method. These assays gave a final lipid percentage of around a quarter of the dry weight being lipid, which is in line with literature (Figure 22). All treatments produced similar levels of lipids.

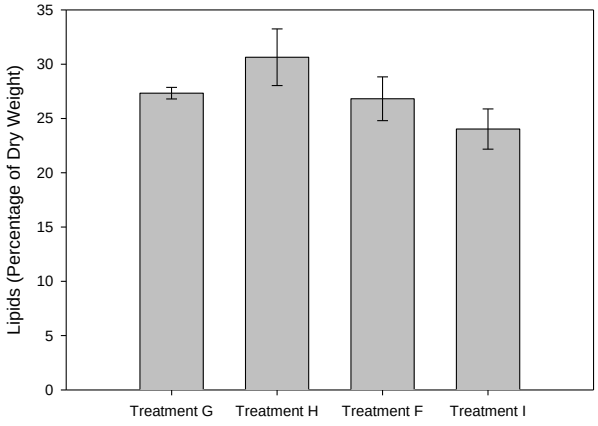

Figure 22: Lipid content in treatments F-I. The percentage of dry weight *C. vulgaris* that was lipid for Treatments F-I.

3.6.3 Protein

Microalgae are also grown for their protein content, as algae can serve as feed for aquaculture and livestock. For instance, intracellular protein may comprise as much as 60% of the dry weight of *Chlorella*, an alga that has an amino acid composition which is similar to animal protein (Kotrbáček et al., 2015). Currently, fishmeal is the primary source of protein for aquaculture feed, but is increasingly expensive as demand for seafood grows (Chen et al., 2015). A *Chlorella vulgaris* strain native to China has been grown as a potential alternative protein source, as it has a comparable protein composition to fishmeal, and 70% of the proteins assayed in a study of this strain contained essential amino acids (Chen et al., 2015). Microalgae are also a source of more specialized proteins, such as phycobiliproteins which are desired for their claimed health benefits and ability to serve as food colorants (Vaz et al., 2016). As such, it was determined that an analysis of the protein content of microalgae grown under CGDM conditions was important. Such analyses were performed on *C. vulgaris* grown under treatments F and I, and compared to algae grown in treatments G and H.

Protein was measured using both the Smith and Bradford Assays, but these gave inconsistent results. Therefore a method by Slocombe, based on the Lowry Assay and developed specifically for microalgae—which are sometimes difficult to lyse on account of their cell walls—was used to obtain the protein measurements for *C. vulgaris* described herein (Slocombe et al., 2013).

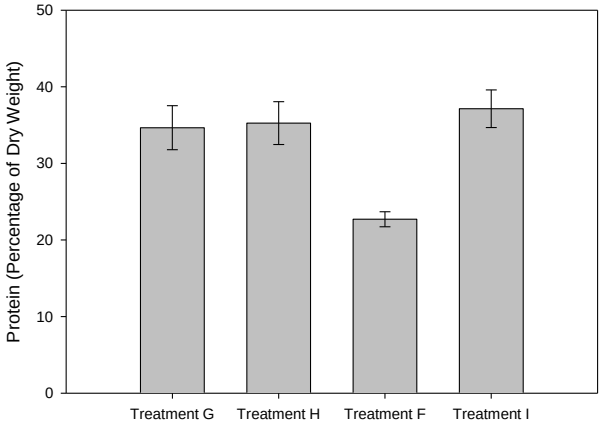

Figure 23: Protein content for treatments F-I. The percentage of dry weight *C. vulgaris* that was protein. There was no large difference between treatments G, H, and I. Treatment F was cultivated for approximately 13 days, during which the percentage of protein may have decreased due to nitrogen depletion.

As illustrated in Figure 23, around a third of the dry weight was estimated to be protein, regardless of treatment, with the exception being treatment F. Treatment F was carried out longer than seven days, and it is conceivable that during this period of nitrogen depletion some quantity of protein was converted into lipid or carbohydrate or other molecules needed for cellular maintenance. For treatments G, H, and I, there was no notable difference in protein level. Thus, there is strong evidence that protein content is also largely unaffected by cultivation in the CGDM-PBR compared with traditional methods of cultivation.

3.6.4 Pigments

Microalgae produce pigments that are valuable as dyes, food colorants, vitamins, antioxidants, anti-inflammatories, nutraceuticals, and pharmaceuticals (Vaz et al., 2016). *D. salina* is grown for its ability to synthesize large amounts of β-carotene, which—along with other carotenoids such as lutein—it uses as a protectant from solar irradiation and high salt levels. β-carotene can comprise as much as 10% of the dry weight of *D. salina* (Farhat et al., 2011).

Lutein is an antioxidant used commercially as a food dye and may play a role in protecting against age-related macular degeneration (Fu et al., 2014). It is sold as a

supplement for vision and eye health. Lutein is concentrated in the retina, where, along with its isomer zeaxanthin, it forms the macular pigment, and is specifically involved with filtering blue light (Kijlstra et al., 2012).

Chlorophyll is the pigment most responsible for carrying out photosynthesis, the process by which carbon dioxide is converted into sugars, from which the plants can further synthesize other carbohydrates, lipids, and proteins. As such, it plays a crucial role in the production of practically all algal biomolecules as well as the biomass.

Therefore, it was considered beneficial to measure chlorophyll concentration for both *C. vulgaris* and *D. salina*, and the β-carotene and lutein content for *D. salina*.

Figure 24 illustrates chlorophyll concentration as a percentage of dry weight for treatments F-I growing *C. vulgaris*. Treatments F, H, and I all had chlorophyll concentrations close to one another, around two percent of the dry biomass. Interestingly, treatment G—the negative control in which atmospheric air was sparged into the PBR—had the highest concentration of chlorophyll, which was different than that of the other treatments. It is not readily apparent why this would be the case, however the concentration of algae for treatment G at the end of the week-long period was less than that of the other treatments and nitrogen was not depleted (Figures 16, 18). Although treatments F, H, and I did not show signs of having reached senescence (Figures 11, 18), it is possible that treatment G was at a stage of growth that had a higher demand for chlorophyll than for the other treatments, and there was still sufficient nitrogen in the medium to synthesize more chlorophyll.

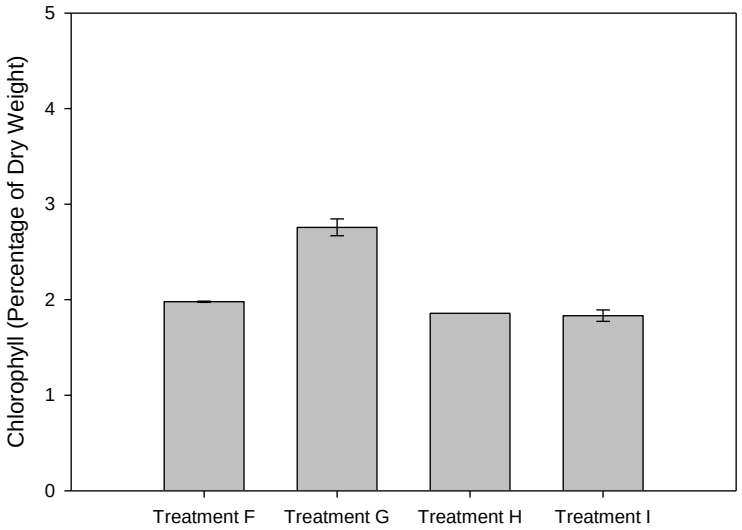

Figure 24: Chlorophyll concentration for treatments F-I. Given as a percentage of *C. vulgaris* dry weight after seven days of cultivation. Treatments F, H, and I all had similar concentrations. Treatment G had a notably larger concentration of chlorophyll compared to each of the other treatments.

For *D. salina*, there was a discrepancy of more than two percent between algae grown at 0.15 LPM and 0.5 LPM, as can be seen in Figure 25, and this difference was significant ($p < 0.01$). At a lower sparging rate, there was a considerably higher chlorophyll concentration as a percentage of dry weight than for algae grown at 0.5 LPM. As with *C. vulgaris*, the biomass for the run at 0.15 LPM was less than that for the one at 0.5 LPM after seven days (Figure 12), so there may have been a similar effect going on as for treatment G vis-a-vis the other *C. vulgaris* treatments. However, in this case, there is also the potential for a self-shading effect by the cells grown at 0.15 LPM compared with those grown at 0.5 LPM. Agitation of the culture in the CGDM-PBR is a result of both mixing by the axial flow impeller as well as sparging. Consequently, at 0.15 LPM, agitation of the culture was less than for 0.5 LPM as the sparging rate was more gentle at the slower rate. Shaded cells would increase chlorophyll in order to capture more photons of light and increase their photosynthetic rate.

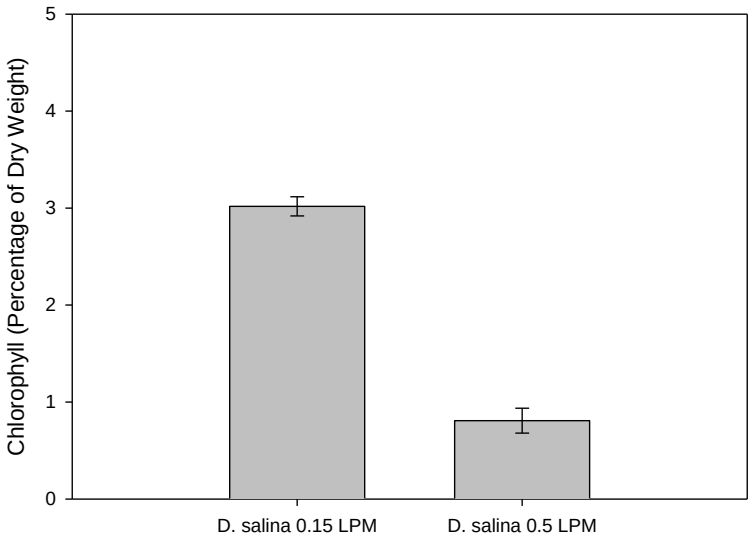

Figure 25: Chlorophyll concentration for *D. salina*. Given as a percentage of dry weight for *D. salina* grown in the CGDM-PBR with sparging rates of 0.15 LPM and 0.5 LPM after seven days of cultivation.

Total carotenoids for *D. salina* were also estimated by the spectrophotometric measurements taken as described in the Materials and Methods (Figure 26). The culture grown at 0.15 LPM produced more carotenoids as a percentage of its biomass than the *D. salina* grown at 0.5 LPM. Although the lighting was 300 μmol m^{-2} s^{-1}, and the NaCl concentration was 1.5 M, no visible carotenogenesis occurred, as is reflected by the total carotenoids for both treatments of *D. salina* comprising less than one percent of the dry weight. Apparently, there was insufficient salt and photo-oxidative damage to trigger a high degree of carotenoid synthesis, despite several studies achieving high carotenoid accumulation under similar or even milder conditions (Çelekli et al., 2014; Wu et al., 2016). However, other studies obtained carotenoid values generally in line with that observed in this study (Fu et al., 2013, 2014). In fact, carotenoid content was found to drop at a light intensity of 225 μmol m^{-2} s^{-1} using a red light LED (Fu et al., 2013), so it may be that the light intensity was so high that carotenogenesis did not proceed. Indeed, when the algal culture was not pre-acclimated to a high light intensity, extensive photobleaching was observed. Therefore, it was tentatively concluded that the light intensity used was too damaging for cells that were not adequately photo-acclimated, but was insufficient to trigger carotenogenesis in cells that were photo-acclimated. To achieve carotenogenesis, one might inoculate *D. salina* grown at a moderate light intensity, but use a lower light intensity in the

CGDM-PBR than 300 μmol m^{-2} s^{-1} such that carotenogenesis occurs, but cells are not stressed to the point of photobleaching and death. An intensity in the range of 150-200 μmol m^{-2} s^{-1} might be used as an initial starting point, balancing between cell growth and carotenoid synthesis (Wu et al., 2016).

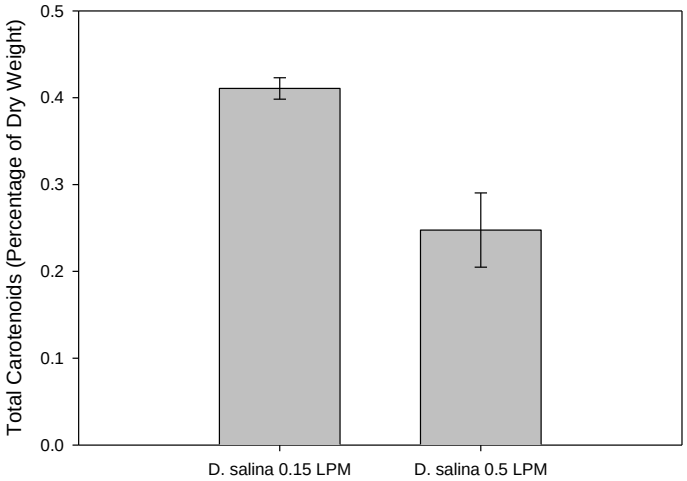

Figure 26: Total carotenoids for *D. salina* grown using the CGDM-PBR with sparging rates of 0.15 LPM and 0.5 LPM.

The chlorophyll a to b ratio, as shown in Figure 27, was also different between the two treatments. This indicates that the *D. salina* in the culture grown at 0.15 LPM had a proportionally higher amount of chlorophyll b than for cells grown at 0.5 LPM, suggesting that for these photo-acclimated cells, there may have been more light-shading for the culture grown at 0.15 LPM than the one grown at 0.5 LPM.

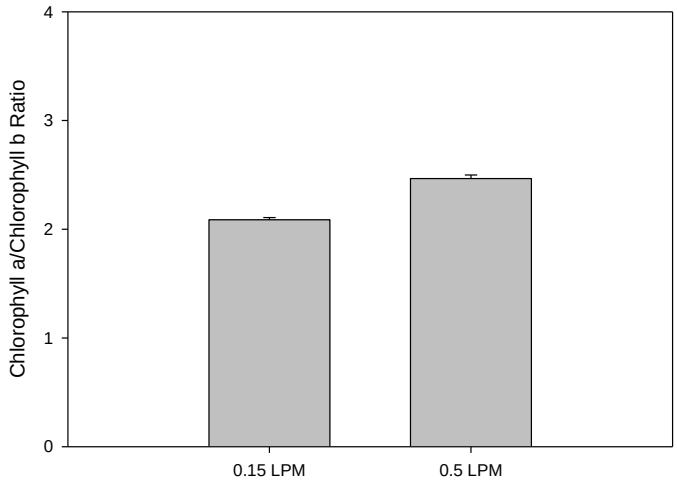

Figure 27: Chlorophyll a to chlorophyll b ratio for *D. salina* grown at 0.15 LPM and 0.5 LPM sparging rates.

Lutein percentages of algal samples for treatments involving sparging at 0.15, 0.5 and 1.0 LPM are shown in Table 3. The amounts measured as a percentage of dry weight were at the low end compared to those found in other studies (Fu et al., 2013; Hu et al., 2008). One explanation for this was the lack of carotenogenesis during the course of these runs, without carotenogenesis, the amount of lutein synthesis—as well as the synthesis of other carotenoids—would be less than that had carotenogenesis been instigated.

Table 3: Lutein content in *D. salina* samples for cells grown at 0.15 LPM and 0.5 LPM. Error is in standard deviations.

Sparging Rate (LPM)	Lutein (% Dry Weight)	Lutein (% Carotenoids)**
0.15	0.0342 ± 0.0010	1.0082 ± 0.0304
0.5	0.0272 ± 0.0039	1.1403 ± 0.1633

**The term 'carotenoids' refers to the material remaining after the extraction process.

RP-HPLC analysis of the β-carotene extract did not resolve clear peaks with which to create a standard curve relating peak area to β-carotene concentration. The β-carotene supplement from which the standards were made was more than two years past its expiration

date. Without a standard curve, the quantities of β-carotene in the algal samples were not estimated.

3.7 Gas Transfer of Carbon Dioxide into the Algal Culture

To confirm that CO_2 could transfer into the CGDM-PBR in a rapid fashion, we measured the time it took for oxygen to saturate an oxygen-depleted *C. vulgaris* algal culture at the end of treatment I in order to measure the k_La, or mass transfer coefficient times the interfacial surface area between the gas and liquid phases. To limit oxygen production as a byproduct of photosynthesis, lighting was turned off prior to the start of testing. Oxygen was displaced from the medium with nitrogen gas. For further confirmation, we performed a similar procedure in tap water. Both algal and tap water tests were done in duplicate.

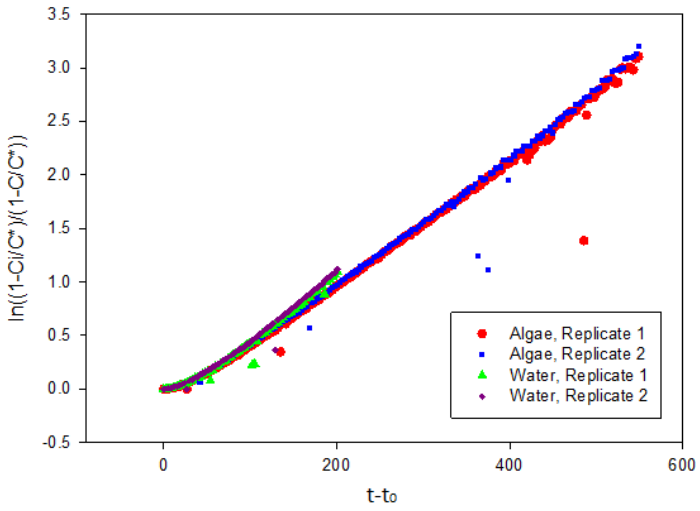

Figure 28: Graph used to calculate k_La. Only linear range shown.

Figure 28 shows that a graph of time (the recorded time minus the initial time, which was taken to be zero) versus $\ln[\frac{1-\frac{Ci}{C^*}}{1-\frac{C}{C^*}}]$ is linear over the beginning portion of the measured time, and that the slope of the resulting linear regression line may be used to estimate the k_La of oxygen into the CGDM-PBR filled with 500 mL of algal culture or tap water. The values collected are shown in Table 4, with the k_La for CO_2 being calculated as 91% of the values for the corresponding k_La for O_2 (Kordač and Linek, 2008).

Table 4: Calculated k_La for oxygen and carbon dioxide in the CGDM-PBR using algal culture and water

Test	k_La (O_2)	Average	k_La (CO_2)
Algae, Replicate 1	0.0058 s^{-1}		
Algae, Replicate 2	0.0060 s^{-1}	0.0059 s^{-1}	0.0053 s^{-1}
Water, Replicate 1	0.0058 s^{-1}		
Water, Replicate 2	0.0056 s^{-1}	0.0057 s^{-1}	0.0052 s^{-1}

3.7.1 Effect of the Cylindrical Containment Chamber on Gas Transfer

In order to gain some sense as to what effect the cylindrical CO_2 containment chamber, which also served as a baffle of sorts, had on the transfer of CO_2 to the algal culture, k_La measurements were performed as described above, but in 500 mL of deionized water, at differing sparging rates (0.4, 1.0, 1.7, and 2.0 LPM) using CGDMs possessing or lacking the cylindrical chamber. Tests were performed in triplicate. As can be seen in Figure 29, the sparging rate and the presence of the chamber, or baffle, did effect the rate of gas transfer, as reflected by the k_La. Gas transfer was generally faster at a higher sparging rate, and with the cylindrical chamber. One hypothesis to explain part of why the chamber enhanced the mass transfer of CO_2 into the liquid phase is that by containing the CO_2 as well as the bubbling in a small volume, the containment chamber increased the gas-liquid interfacial area in this portion of the PBR, leading to an increase in gas transfer that more than compensated for the fact that not all the liquid medium (water in this case) was directly exposed to the sparged gas.

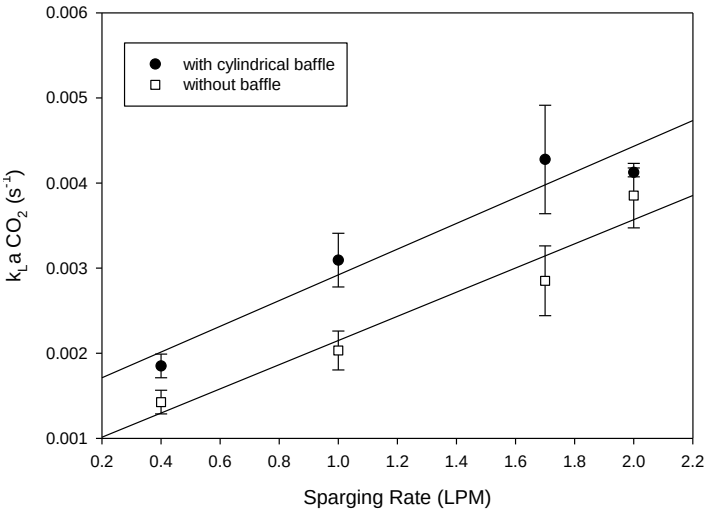

Figure 29: Graph of k_La with and without cylindrical baffle. Measured in 500 mL of deionized water.

3.8 Carbon Dioxide Fixation Efficiency

As anticipated, demand for bicarbonate for CO_2 generation increased in line with algal growth in treatment I for the first four days (Figure 30). Although algal growth was maintained at exponential phase up to the seventh day, demand for carbon via carbon dioxide plateaued at around 7.8 mmol/day.

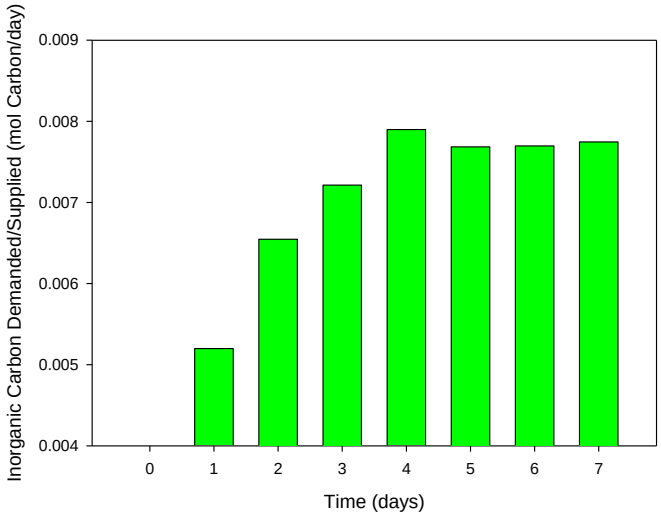

Figure 30: Moles of carbon supplied to the CGDM-PBR in treatment I. In response to pCO_2 levels dropping to 0.25%.

The CGDM-PBR system led to a high carbon fixation efficiency of 75.4% ± 2.4% in treatment I, drastically higher than treatment H, in which air enriched with 0.435% CO_2 was constantly sparged into the culture as a positive control (Figure 31). It was also more efficient than the negative control, treatment G, in which air with an assumed pCO_2 of 0.04% was sparged into the system.

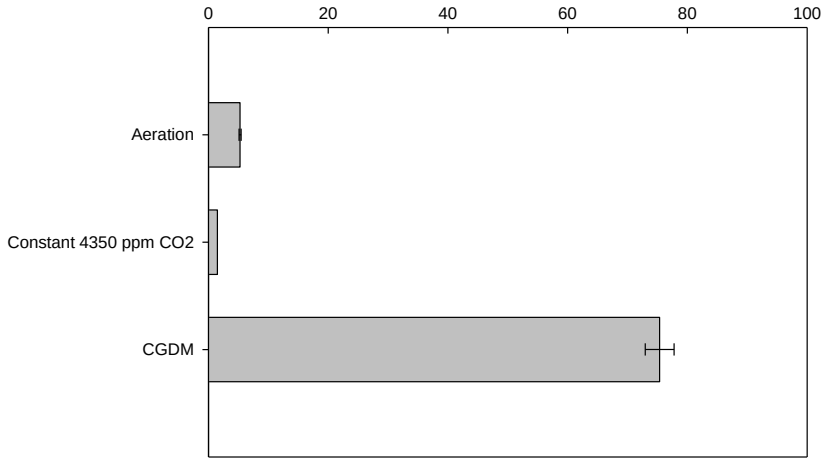

Figure 31: The carbon fixation efficiencies after seven days. For treatments G, H, and I, representing a negative control, positive control, and test run of the CGDM, respectively.

In the initial days of cultivation, the efficiencies were lower, reflecting that more CO_2 was being supplied than the relatively low number of algal cells required. Presumably, this CO_2 was lost to leakage from the system. As cell density increased, fixation of carbon became more efficient (Figure 32).

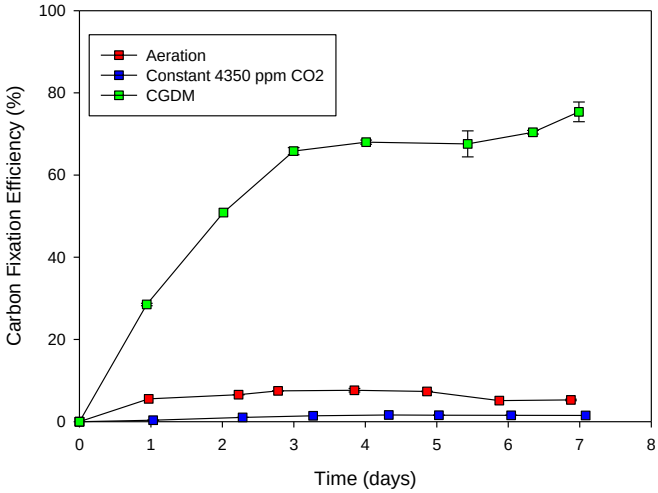

Figure 32: Timecourse of carbon fixation efficiencies by day of cultivation for treatments G, H, and I. (A) Aeration of atmospheric air, (B) constant sparging with CO_2-enriched air with a pCO2 of 4,350, and (C) the CGDM set to generate and cease generation of CO_2 at 0.25% and 0.5% pCO_2.

The carbon fixation efficiency was also measured for *D. salina*, as is shown in Figure 33. At 0.5 LPM, an efficiency of 62.5% ± 0.2% was observed, while an efficiency of 43.7% ± 0.1% was observed for the run at 0.15 LPM. A gas-sampling pump (NMP 09 M) had to be used to produce a sparging rate of 0.15 LPM, as the original NMP 830 KNDC B model diaphragm pump had a minimal bubbling rate of around 0.5 LPM. The lower efficiency of the 0.15 LPM run may have been due to the use of this different air pump, which may have resulted in more leakage of gas outside of the CGDM-PBR system, as leak tests indicate (Figure 34). Presumably the efficiency would have been higher with a more hermitically sealed pump. Furthermore, different algal species have been found to have different carbon fixation efficiencies (de Morais and Costa, 2007). Still, both runs indicate that the CGDM is capable of achieving carbon fixation efficiencies much higher than that obtained by traditional methods, especially when the CGDM system is airtight. For a general comparison, open-culture microalgal cultivation typically has a carbon fixation efficiency of 10-30% (Li et al., 2013). Even in a closed photobioreactor system, the fixation efficiencies are often not high, with an efficiency of 58% recorded for a semi-continuous photobioreactor (Chiu et al., 2008). One study found an inverse correlation between the concentration of CO_2 and the fixation efficiency, with greater efficiency at lower CO_2 concentrations (de Morais and Costa, 2007).

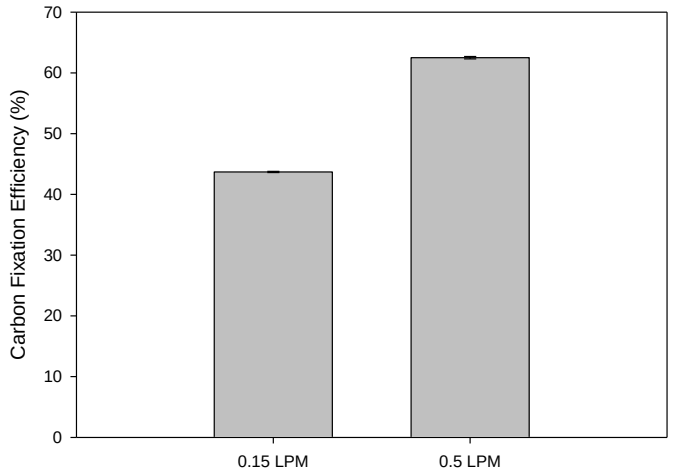

Figure 33: Carbon fixation efficiency of the CGDM-PBR growing D. salina at sparging rates of 0.15 LPM and 0.5 LPM

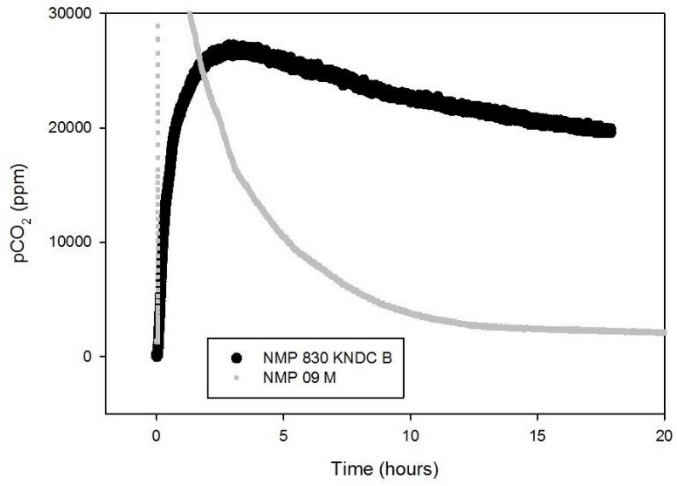

Figure 34: Leak test of the CGDM-PBR system using the diaphragm pump and micro diaphragm pump. CO2 was introduced to the system, which was then closed and the pumps used to recirculate gas within the system.

Furthermore, the solubility of CO_2 is inversely correlated with salinity (Al-Anezi et al., 2008). The modified Pick's Medium in which *D. salina* was cultivated had a NaCl molarity of 1.5. At this high of a salinity, the carbon dioxide solubility is estimated to be less than 60% that of salt-free water (Chang et al., 1998; University of Kansas, 2003). The less carbon dioxide that can be dissolved into media for a given pCO_2 in the headspace of the PBR, the less CO_2 there is available to be taken up by the algae. This lower solubility may

have contributed to the small disparity in carbon fixation efficiencies between CGDM cultivations of the freshwater *C. vulgaris* and *D. salina*.

CHAPTER 4: CONCLUSION AND FUTURE WORK

4.1 Synopsis of Findings

The studies detailed in this thesis have described a means by which photosynthetic microalgae may be cultivated in such a way that carbon dioxide is conserved with low degree of wastage.

It was found that for the optimal operation of the CGDM, constant sparging of the CO_2-enriched gas was required. Convective diffusion solely due to mixing by the axial flow impeller and thermodynamic equilibrium is insufficient for the mass transport of CO_2 to the algal cells in a timely manner, and for the removal of wastes such as oxygen from the cells.

Urea was shown to be an excellent alterative to nitrate for both *C. vulgaris* and *D. salina* cultures. Urea provides twice as much nitrogen per mole as nitrate, and did not contribute to a large change in pH the way nitrate would. As long as urea is not added to culture in toxic amounts, and the microalga to be cultured possesses urea carboxylase and allophanate lyase or equivalent enzymes, it is a very viable substitute to nitrate salts for the purpose of supplying nitrogen to microalgal cultures.

The experiments demonstrated that a relatively low concentration of CO_2 was needed per dosage of the CGDM to achieve good results. Using lower concentrations per dosage should also reduce the amount of CO_2 lost to leakage, particularly if the CGDM were to be used in a more open system where dissolved CO_2 could escape into the atmosphere due to molecular equilibrium.

The CGDM was tested at varying sparging rates and impeller speeds using two microalgal species, one a non-motile freshwater alga with a cell wall, the other a wall-less flagellate grown in hypersaline medium. The CGDM proved capable at growing both to a high density.

4.2 Future Work

There remains much more to learn about the CGDM, and how it can be improved upon. The CO_2 concentration per dosage can be decreased further, and the system can be more robustly sealed to prevent leakage of gas into and out of the CGDM-PBR apparatus. This should allow for an even greater carbon fixation efficiency.

Due to time and resource constraints, only one run of the CGDM under each treatment was performed, and consequently good biological replicates were lacking. More

biological replicates, especially under the conditions found to be conducive of ample growth, might be performed in future.

The CGDM was designed with the goal that it might be used commercially and modularly. As such, the CGDM might be tested in a raceway pond setting, with individual CGDMs spaced around the raceway. The carbon fixation efficiency will undoubtedly drop due to dissolved CO_2 dissipating out of the culture in order to reach equilibrium with the atmosphere, but the CGDM will likely be a marked improvement on current methods of sparging CO_2 into the culture—which wastes considerable CO_2—or relying on ambient CO_2, which results in slower growth.

C. vulgaris was grown photoautotrophically, with CO_2 forming the sole carbon source. *D. salina* was grown in medium containing a relatively small amount of sodium bicarbonate, primarily to serve as a pH buffer. If cultivated mixotrophically, with an organic carbon source from wastewater, for instance, it is highly conceivable that the CGDM can grow algae to an even higher density than demonstrated here.

For the CGDM experiments described herein, carbon dioxide was generated by the reaction of sodium bicarbonate and hydrochloric acid. However, the CGDM can use CO_2 produced by reactions of several bicarbonate or carbonate salts with an appropriate acid. Research can be conducted on the effectiveness of alternative acid and bicarbonate/carbonate combinations, in regards to the running and costs of operation of the CGDM.

The biomolecular composition of microalgae can be altered based on culture conditions, including medium makeup, lighting, temperature, shear stress, etc. (Chen et al., 2012; Fu et al., 2013, 2014; Kokkinos et al., 2016; Liang et al., 2009; Rosenberg et al., 2014; Wu et al., 2016). Depending on the product of interest, culture conditions should be modified to optimize the synthesis of that product. All of these factors can be tested further with the CGDM-PBR system in differing configurations.

In the experiments in this thesis, pCO_2 was read by measuring the CO_2 concentration in the headspace of the CGDM-PBR system. This was due to the equipment available and constraints based on the physical size of the CGDM-PBR. Rather than CO_2 in the air above the culture, it is more important to know the dissolved concentration of carbon dioxide in the medium, as this is the carbon that will be taken up by the algae. Therefore, future research could benefit from a direct measurement of the dissolved CO_2 and other inorganic carbon concentrations in the medium.

Additionally, the CGDM was designed based on being triggered by a lower pCO_2 measurement and then being turned off by an upper pCO_2 reading. However, this led to a small degree of overshoot in CO_2 generation. This overshoot might be reduced, if not eliminated, if the CGDM was re-programmed to be triggered by an arbitrary pCO_2 reading as before, but was set to generate CO_2 for a set amount of time. In this fashion, the amount of CO_2 generated per dose could be more accurately produced, and would not rely on the CO_2 sensor measuring a set upper value. This would remove excess CO_2 generated as a result of a delay between CO_2 being generated, passing to the CO_2 sensor chamber, and then being recorded by the sensor. Still, if such a re-programming were to take place, it would be wise to produce enough CO_2 per dosing to ensure adequate carbon was delivered to the algae.

4.2.1 Self-Aspirating Impeller

One proposed idea for a modification to the CGDM is to use a self-aspirating impeller to replace the axial flow impeller used in the studies detailed here (Murthy et al., 2008). Primarily for the use of the CGDM on a larger scale in a commercial, raceway pond, setting, this would allow for a reduction in cost due to the purchase an operation of an air pump to sparge CO_2 into the algal medium (Figure 35). To produce sufficient bubbling, the impeller will likely have to be run at a high speed, therefore such a design would be best suited to hardy microalgae such as *C. vulgaris* that possess a cell wall and lack flagella.

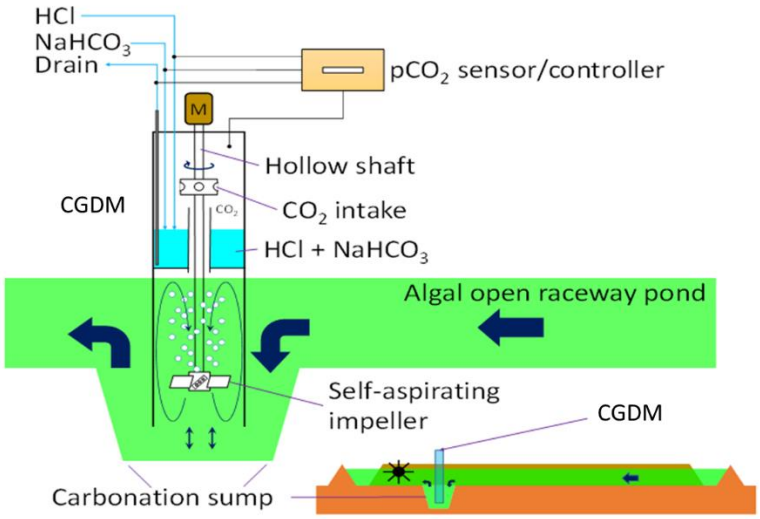

Figure 35: A conceptual design of a CGDM utilizing a self-aspirating impeller. For the cultivation of microalgae in a raceway pond.

4.2.2 Carbonic Anhydrase

Studies into the design and operation of the CGDM grew out of research of the enzyme carbonic anhydrase and its potential use in temporary carbon dioxide fixation. Carbonic anhydrase is an enzyme catalyst capable of interconverting inorganic carbon between carbon dioxide and bicarbonate ions by the following net reaction (Berg et al., 2002):

$$CO_2 + H_2O \xleftrightarrow{Carbonic\ Anhydrase} [H_2CO_3] \rightleftharpoons HCO_3^- + H^+$$

The reverse reaction from bicarbonate to carbon dioxide is favoured at neutral pH and lower (Berg et al., 2002). The forward reaction of CO_2 to bicarbonate occurs more readily at higher pH, with maximal conversion at around a pH of 9.0 (Berg et al., 2002). *C. vulgaris* has been found to grow optimally in a pH range of 7.5-8.0 (Rachlin and Grosso, 1991). For *D. salina*, the optimal pH for growth is less clear, with one study finding best growth at around 9.0, and another at 7.0, but acknowledging good growth even at 9.0 (Loeblich, 1982; Ying, 2014). Additionally, experiments growing *C. vulgaris* with recombinant carbonic anhydrase resulted in poor growth, potentially due to the enzyme's somewhat promiscuous specificity allowing it to react with other molecules besides carbon dioxide and bicarbonate ion. Bovine carbonic anhydrase, for instance, possesses hydratase activity for a number of substrates (Worthington and Worthington, 2011). Furthermore, carbonic anhydrase contains a ligand-bound zinc ion that is a necessary cofactor for the proper functioning of the enzyme (Worthington and Worthington, 2011). This zinc ion can be abstracted due to competition with phosphorus and sulfur ions which can also chelate this ion (Sun and Wang, 2009). Phosphorus and sulfur are vital ingredients in both Bold's Basal Medium and Pick's Medium, and presumably all microalgal media. Consequently, it was determined that using carbonic anhydrase directly in conjunction with algal culture was unlikely to be effective.

This research, along with other concerns, led to the concept that carbonic anhydrase might be used to simply convert carbon dioxide into an aqueous bicarbonate solution which could then be stored and transported to a site of algal cultivation. As needed, the bicarbonate could be reacted with an acid to produce CO_2 gas which could be sparged into the algal culture. Refinement of this concept resulted in the CGDM.

The recombinant carbonic anhydrase was tested and found to be enzymatically active and capable of converting between CO_2 and bicarbonate. The enzyme was found to be generally stable in phosphorus-free buffer, but prone to rapid degradation when sparged,

particularly when combined with anionic surfactant (various polysorbates and polaxamers) and foamed.

In order to try to increase stability of the enzyme over time, carbonic anhydrase was coated on silica beads following the protocol of Neville, et al. using polyethylenimine of molecular weights 10,000 and 70,000 (Neville et al., 2011). Additionally, two other recombinant protein enzymes, UHCA and UHCCA, were used to coat silica beads. UHCA is a protein containing linked domains of URE, a hydrophilic domain, and carbonic anhydrase. UHCCA is a recombinant enzyme comprised of linked domains of URE, a hydrophilic domain, cohesin, and carbonic anhydrase. The beads were stored in phosphorus-free NaCl buffer (pH of 7.5) and their activity measured over the course of several days using the Wilbur-Anderson assay, a common assay to measure carbonic anhydrase enzymatic activity based on the time needed for the pH of a CO_2 solution to drop from 8.3 to 6.3.

As illustrated in Figure 36, all carbonic anhydrase containing beads lost activity over the course of a few days, with the largest drop occurring within the first day, but after this initial period the activity remained relatively stable.

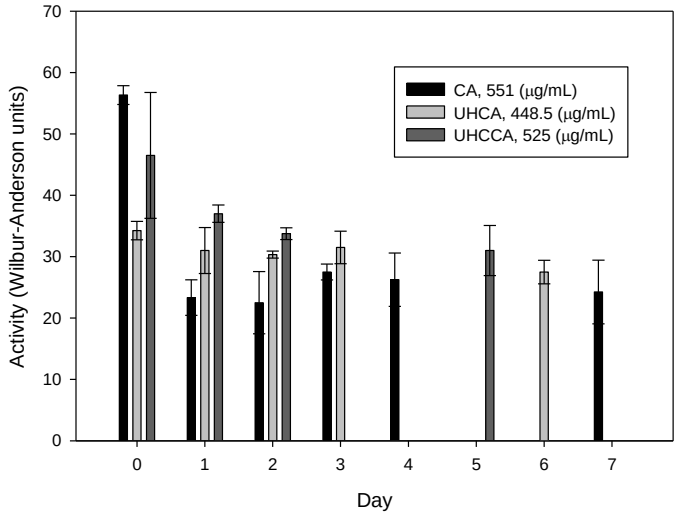

Figure 36: Activity of carbonic anhydrase and variants coated on silica beads over time. Concentrations are net concentrations of the enzyme on beads, i.e. 551 μg carbonic anhydrase on one milliliter of beads. (CA: carbonic anhydrase; UHCA: ure-hydrophilic domain-carbonic anhydrase; UHCCA: ure-hydrophilic domain-cohesin-carbonic anhydrase.)

If CO_2—from flue gas, for instance—is gently sparged into a high pH phosphorus-and-sulfate-free buffer containing carbonic anhydrase coated beads, bicarbonate solution will be formed and this solution can then be stored elsewhere and transported to an algacultural site. The enzymatic beads can be reused, but will likely have to be replaced around weekly based on the findings shown above or more frequently depending on extent and duration of agitation of the enzymes. This would help reduce greenhouse gas emissions and produce a cheap bicarbonate solution for the CGDM. Figure 37 shows a calibration curve created for the purpose of measuring enzyme-coated silica beads spectrophotometrically.

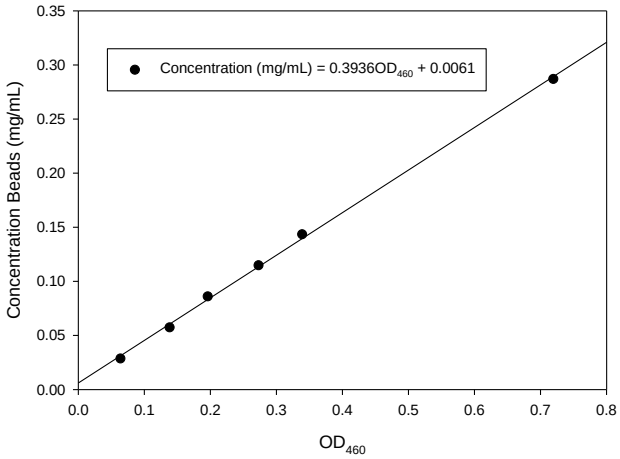

Figure 37: Calibration curve relating carbonic anhydrase thiol bead concentration to absorbance at 460 nm. Carbonic anhydrase thiol beads were formed with MW 70,000 PEI and 8.7 mg mL^{-1} carbonic anhydrase.

4.2.3 Miniaturization

The CGDM can also be miniaturized and used as a convenient means of portable CO_2 supply for lab cultures in instances where carbon dioxide gas tanks are not a feasible option due to size, weight, or hazard constraints, etc. For such purposes, it would likely be best for the impeller to be driven from a motor attached to the CGDM rather than by a magnetic stirrer.

4.3 Final Words

The culmination of this research has led to the design, construction, and operation of the CGDM, which has been shown to be a valuable means of achieving high photoautotrophic microalgal biomass growth while conserving carbon dioxide. The general biomolecular composition of the algae has been demonstrated to be similar to algae grown

under standard commercial and laboratory regimens, supporting the viability of using the CGDM for laboratory and commercial microalgal cultivation. Future research can help refine and modify the CGDM system and elucidate additional means of maximizing microalgal product yield while reducing the amount of carbon dioxide needed and other contributors to the costs of operation in algaculture.

CHAPTER 5: REFERENCES

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APPENDICES

Appendix I: Photographs

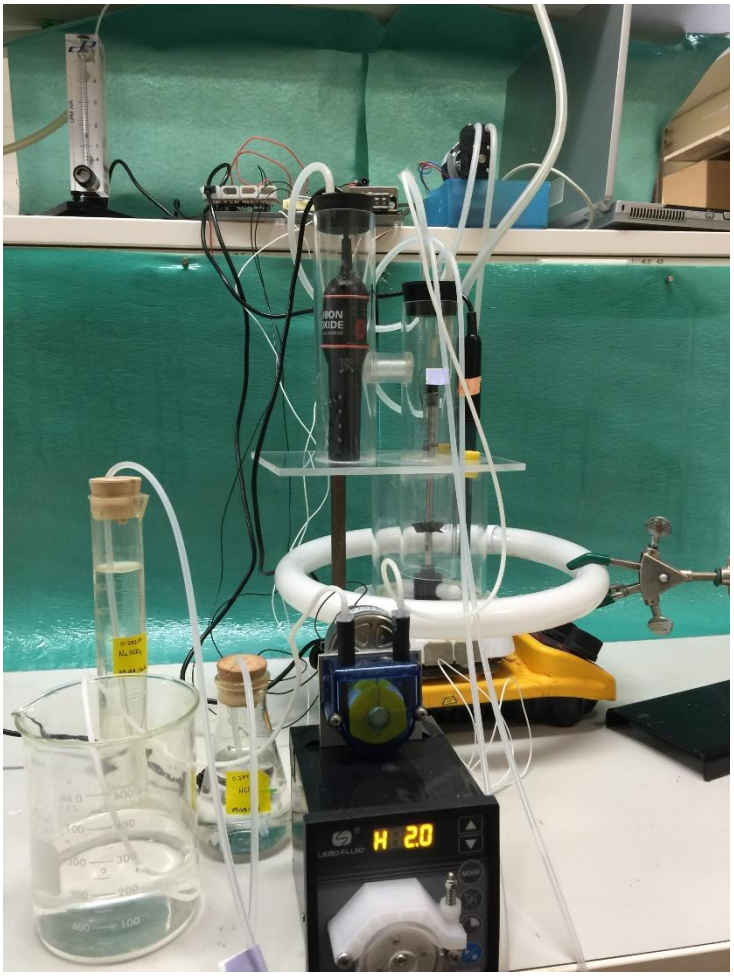

Figure A1: Photograph of the CGDM-PBR with a single toroidal fluorescent light, Vernier pH and CO_2 sensors, $NaHCO_3$ and HCl solutions, liquid pump set to deliver ~0.33 mL min^{-1}, diaphragm air pump (in blue container at upper right), and Arduino circuitboard on shelf to the upper left of the CO_2 sensor with Vernier jack fixture.

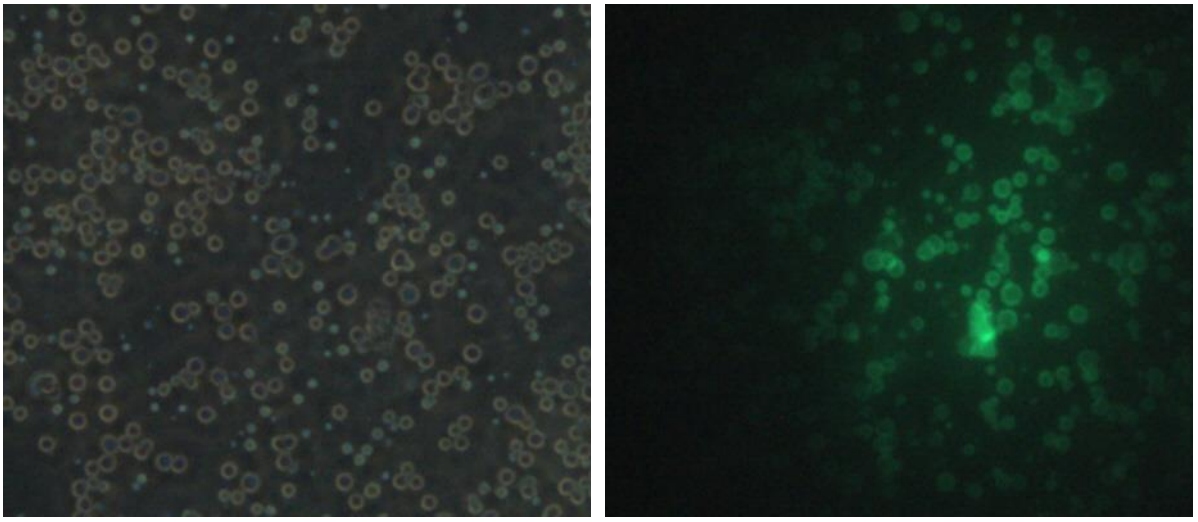

Figure A2: Silica beads coated with green fluorescent protein (GFP). Left: Beads as they appear without noticeable fluorescence. Right: Beads with GFP fluorescing.

Appendix II: Chromatograms

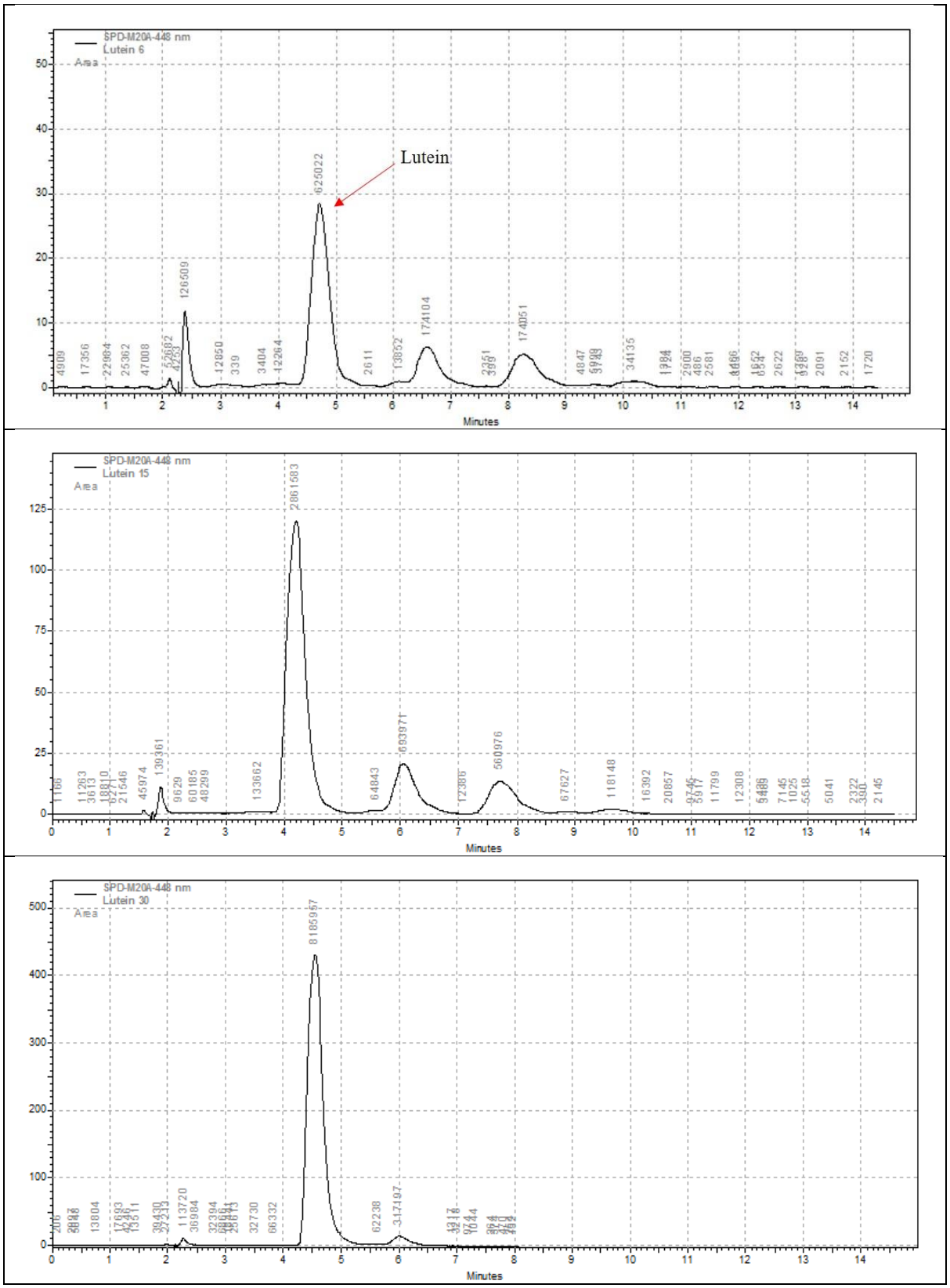

Figure A3: RP-HPLC chromatograms used to construct lutein standard curve. Top: Six µg mL^{-1} lutein. Middle: 15 µg mL^{-1} lutein. Bottom: 30 µg mL^{-1} lutein.

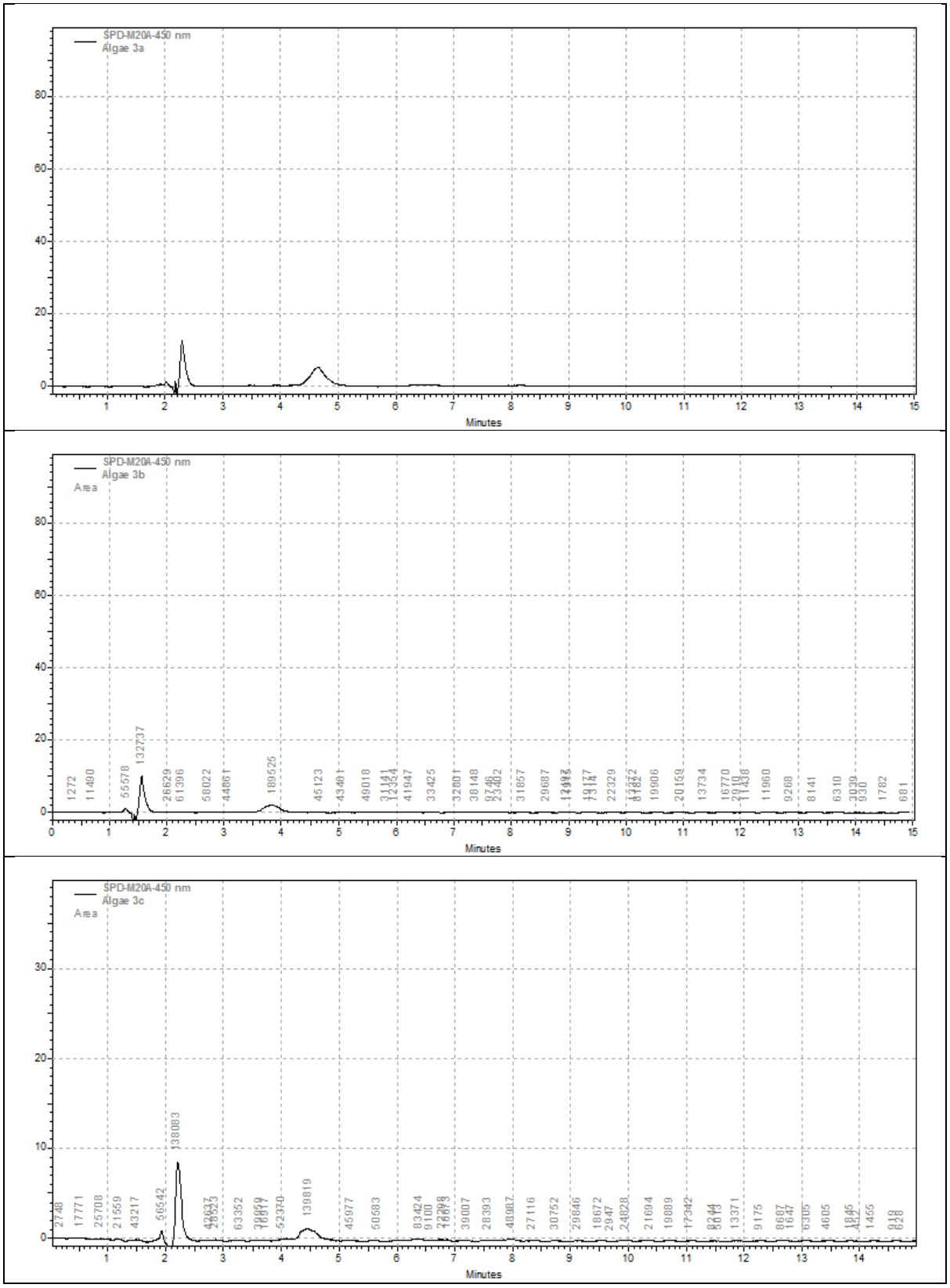

Figure A4: RP-HPLC chromatograms used to calculate concentration of lutein in D. salina grown in the CGDM-PBR with sparging at a rate of 0.15 LPM

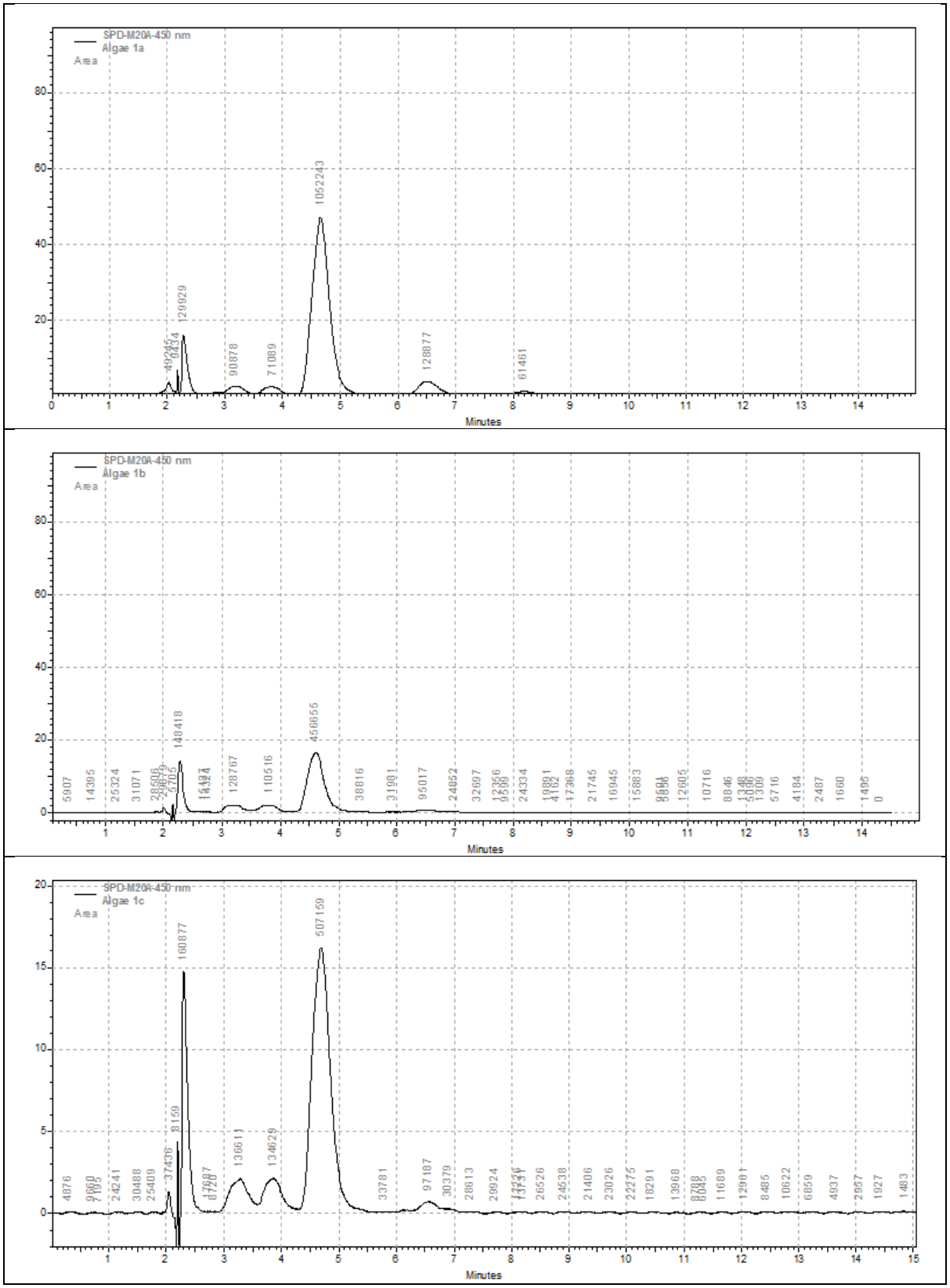

Figure A5: RP-HPLC chromatograms used to calculate concentration of lutein in D. salina grown in the CGDM-PBR with sparging at a rate of 0.5 LPM

Appendix III: Protocols and Standard Operating Procedures

Standard Operating Procedure
for
CO2 Sensing and Dosing Arduino Program

1. Obtain USB cable, one end Type A USB, the other end Type B. Attach Type A end to computer with Arduino and program. Attach Type B end to Arduino circuit board.
2. Obtain Vernier CO_2 sensor probe and insert probe into gas sensing chamber. Seal with rubber stopper designed to wrap around cable. Attach the probe's cable to the leftmost jack on the Arduino (facing the available jacks).
3. Plug in power supply on circuit board apparatus (large collection of motherboards bound to plexiglass) into outlet.
4. Turn on liquid pump by plugging in cable to outlet. H 2.0 is approximately 0.33 mL/min.
5. On computer, open Arduino program, "_20160419_co2".
6. In the menus at the top of the window, select Tools > Ports > "COM9 (Arduino Uno)".
7. If necessary, ensure that Tools > Board > "Arduino Uno" is selected.
8. In the program, use "#define CO2MIN" (without quotes) as the value in ppm of your choosing (e.g. 2500 = 2500 ppm CO2). Do the same for #define CO2MAX.
9. Under ISR (TIMER1_COMPA_vect) section where it is written "if (seccount == ####)" where #### is some number, change recording interval to the time interval in seconds you would like to record the CO_2 ppm readings, e.g. 600 = 600s = 10 min (the pCO_2 is recorded every 10 min as ppm).
10. To change the time in which the system drains waste CO_2 generation fluid, change "#define PUMPONTIME" to the value of your choosing in milliseconds (e.g. 30000 would be 30 seconds).
11. Upload program (button with right pointing arrow). You should hear a click from the Arduino circuit board and a flash from a blue LED.
12. Open CoolTerm.exe.
13. Select Options > Serial Port and pick COM 9 from the dropdown menu. Press "OK." Select Re-Scan Serial Ports if COM 9 is not available, and make sure USB cable is plugged in.
14. Select Connection > Capture to Textfile > Start. A window should pop up, requesting a name for file.
15. Name the file what you would like (in .txt format), and in the directory of your choosing. Save.
16. Select the connection button on the main screen (with the USB cable icon). You should hear a click and a blue LED flash from the Arduino, and the system should run.
17. You may open your text file to which you are saving the data periodically to make sure it is doing so.
18. When you are done with the run, in CoolTerm.exe, open Connection > Capture to Textfile and select Stop. This ends the recording of your data.
19. Select Disconnect from the main screen in CoolTerm.exe and shut down.

20. Close Arduino.
21. Unplug USB cable connecting Arduino to computer. Unplug liquid pump. Unplug power source to circuit board apparatus.
22. Put away Vernier sensor if you are not using in the near future.

Standard Operating Procedure
for
Measuring Light Intensity

Materials

- Light sensor with cable – make sure wires are connected
- USB cable
- Light sensor box (EME Systems) with memory card inserted and depressed
- Computer with Tera Term software

Procedure

1. Assemble light sensor by attaching light sensor to light sensor box, if not already attached. Either cable port on the box will work. Attach USB cable to box and to computer. Insert memory card into box if it isn't already inserted.
2. Open Tera Term software.
3. When pop-up screen appears, select "Serial" and choose "COM 7: USB Serial Port" (or the one with "COM 7," not COM 3). Press OK.
4. In dropdown Control menu at the top of the main window, select "Macro." A pop-up window will appear to let you choose a macro.
5. Select senddtrl.ttl
6. A menu should appear on the main screen. Enter 'R' (without quotes, no need for capitalization) to run.
7. The light intensity values are on the right of the string of numbers. Look at legend for identification of (other) numbers.
8. You can change the time interval if needed. See legend on screen for instructions.
9. When done, close Tera Term, and disconnect and disassemble light sensor apparatus. You can keep the memory card, light sensor, and cable attached to the sensor box. The USB cable can be kept with the light sensor apparatus if it is only used for that purpose. Otherwise, put in data cable drawer. Shut down computer if not using.

Troubleshooting

- Check that cable wires are attached properly to the green connector component on the light sensor cable. They should be well-glued on, and this shouldn't be an issue now
- Make sure that the memory card is inserted and depressed in the sensor box
- Select COM 7, not COM 3
- The right side USB ports on the VAIO laptop are more reliable than the one on the left